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New insights into biological effects of conjugated linoleic acid and saturated fats in body fat composition, obesity and related disorders:

Experimental studies in normal-weight Wistar and obese Zucker rats

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ABSTRACT

New insights into biological effects of conjugated linoleic acid and saturated fats in body fat composition, obesity and related disorders: experimental studies in normal-weight Wistar and obese Zucker rats

The daily intake of conjugated linoleic acid (CLA) isomers by humans, through diet and supplementation, and the controversial effects of these compounds in human health, were the main motivation for the elaboration of this thesis. Firstly, the present work intended to estimate the daily CLA ingestion by the Portuguese population. Secondly, the biological effects of CLA were exploited using two distinct animal models, normal-weight (Wistar rat) and genetically fat (obese Zucker rat), in combination with saturated fat based diets. The estimative of total CLA intake for the Portuguese population was 73.70 mg/day. The *cis*(*c*)9,*trans*(*t*)11 and *t*7,*c*9 were the most prevalent CLA isomers, with, respectively, 76.10 and 12.56% of the total CLA intake value. Concerning the animal trials, CLA in conjugation with saturated fats revealed beneficial but also deleterious biological effects. In the normal-weight Wistar rat fed a palm oil based diet, the administration of *c*9,*t*11 CLA isomer increased the serum triacylglycerols and the size of adipocytes from epididymal and retroperitoneal fat depots. In addition, a CLA mixture of *c*9,*t*11 and *t*10,*c*12 isomers increased the glycerol membrane permeability of kidney proximal tubules, which may indicate an improvement of glycerol reabsorption pathway. In the obese Zucker rat, CLA (as a mixture) induced changes in fatty acid profile of liver, muscle and adipose depots. CLA supplemented with a vegetable saturated fat diet seemed to promote a more beneficial adipokine serum profile and an alleviation of hepatic steatosis. In contrast, adverse effects of CLA were observed with hypercholesterolaemia promotion. Regardless CLA, the ovine fat diets worsened the insulin resistance and increased the pro-inflammatory serum cytokines. In the liver, different levels of cell death and apoptotic pathways were modulated by CLA, depending on the type of saturated fat present in the diet. The most striking result of this study was that CLA was not able to promote fat loss in both experimental models. Moreover, new mechanisms of CLA action were disclosed in this work, which reinforce the need to further investigate this compound.

Key words: conjugated linoleic acid; saturated fats; Wistar rat; Zucker rat; body fat composition; obesity; lipid metabolism; adipokines.

RESUMO

Efeitos biológicos do ácido linoleico conjugado e de gorduras saturadas na composição da gordura corporal, na obesidade e patologias associadas: estudos experimentais em ratos Wistar e Zucker obesos

A ingestão diária de isómeros do ácido linoleico conjugado (CLA), através da dieta e da sua suplementação, bem como, os efeitos controversos destes compostos na saúde humana, constituíram a principal motivação para a elaboração desta tese. Numa primeira fase, o trabalho pretendeu estimar a ingestão diária de CLA pela população Portuguesa. Posteriormente, foram avaliados os efeitos biológicos do CLA quando suplementado a dietas à base de gordura saturada. Para tal, recorreu-se a dois modelos animais distintos, o rato Wistar e o rato Zucker (geneticamente obeso). A ingestão média total de CLA pela população Portuguesa foi estimada em 73.70 mg/dia. Os isómeros do CLA mais representativos foram o *cis*(*c*)9,*trans*(*t*)11 e o *t*7,*c*9, correspondendo, respectivamente, a 76.10 e 12.56% do total de CLA ingerido. Quanto aos estudos *in vivo*, o CLA revelou efeitos biológicos tanto benéficos como prejudiciais. No modelo Wistar alimentado com dieta à base de óleo de palma, a administração do isómero do CLA *c*9,*t*11 elevou os níveis de triacilgliceróis no soro, bem como, o tamanho dos adipócitos das gorduras epididimal e retroperitoneal. Adicionalmente, a mistura de isómeros do CLA (*c*9,*t*11 e *t*10,*c*12) aumentou a permeabilidade membranar ao glicerol no túbulo proximal do rim, sugerindo uma melhoria no processo de reabsorção desta molécula. No modelo Zucker, o CLA (como mistura de isómeros) induziu alterações no perfil dos ácidos gordos do fígado, músculo e gorduras epididimal e retroperitoneal. O CLA administrado com óleo de palma promoveu um perfil de adipocinas no soro mais benéfico e melhorou a esteatose hepática. Em contraste, a suplementação com CLA apresentou um efeito hipercolesterolémico. Independentemente do CLA, a dieta rica em gordura de ovinos agravou a resistência à insulina e aumentou as adipocinas pró-inflamatórias no soro. No fígado, diferentes níveis de morte celular e vias apoptóticas foram modeladas pelo CLA em função do tipo de gordura presente na dieta. É de salientar que não se observaram efeitos anti-adipogénicos do CLA em nenhum dos dois modelos animais. Por último, esta tese contribuiu para a descoberta de novos mecanismos de acção dos isómeros do CLA, reforçando a necessidade de continuar a estudar estes compostos.

Palavras-chave: ácido linoleico conjugado; gorduras saturadas; rato Wistar; rato Zucker; obesidade; metabolismo dos lípidos; adipocinas.

LIST OF PUBLICATIONS

This thesis was based on the following publications and manuscripts:

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[1] These co-authors contributed equally for this paper

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LIST OF ABBREVIATIONS AND SYMBOLS

ACC	Acetyl-CoA carboxilase (EC 6.4.1.2)
ALP	Alkaline phosphatase (EC 3.1.3.1)
ALT	Alanine aminotransferase (EC 2.6.1.2)
ANOVA	Analysis of variance
AQP	Aquaporin
AST	Aspartate aminotransferase (EC 2.6.1.1)
ATF-6α	Activating transcription factor-6alpha
BBMV	Brush-border membrane vesicles
Bip	Binding protein
CHOP	CCAAT/enhancer-binding protein homologous protein
CLA	Conjugated linoleic acid
CPT	Carnitine palmitoyltransferase (EC 2.3.1.21)
CoA	Coenzyme A
CRP	C-reactive protein
DM	Dry matter
E_a	Arrhenius activation energy
EC	Enzyme Commission number
ER	Endoplasmic reticulum
FA	Fatty acid
FAME	Fatty acid methyl ester
Fas	Cell death receptor belonging to the tumor necrosis factor family
FAS	Fatty acid synthase (EC 2.3.1.85)
FDA	Food and Drug Administration
FID	Flame ionisation detector
GC	Gas chromatography
GLM	General linear model
GLUT	Glucose transporter
HDL	High density lipoprotein
HOMA-IR	Homeostasis model assessment using the insulin resistance index (Mathews *et al*., 1985)
HPLC	High performance liquid chromatography
HSL	Hormone-sensitive lipase (EC 3.1.1.3)
IL-1β	Interleukine-1beta
IL-6	Interleukine-6
INE	Instituto Nacional de Estatística
JNK	c-Jun NH_2-terminal kinase
LDL	Low density lipoprotein

LPL	Lipoprotein lipase (EC 3.1.1.34)
MCP-1	Monocyte chemoattractant protein-1
MUFA	Monounsaturated fatty acid
NAFLD	Non-alcoholic fatty liver disease
n.d.	Not detected
ns	Not significant
P	Probability
PAI-1	Plasminogen activator inhibitor-1
PCA	Principal component analysis
$\mathbf{P}_f$	Permeability coeficient
$\mathbf{P}_{gly}$	Glycerol permeability
PPAR	Peroxisome proliferator activated receptor
PUFA	Polyunsaturated fatty acid
SAS	Statistical analysis system
SCD	Stearoyl-CoA desaturase (EC 1.14.19.1)
SD	Standard deviation
SEM	Standard error of the mean
SFA	Saturated fatty acid
SREBP-1	Sterol regulatory element binding protein-1
TAG	Triacylglycerol
TFA	*Trans* fatty acid
TNF-α	Tumor necrosis factor-alpha
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VLDL	Very low density lipoprotein
WHO	World Health Organization

INTRODUCTION

Ever since the National Academy of Sciences recognised conjugated linoleic acid (CLA) as the only fatty acid that unequivocally inhibits carcinogenesis in experimental models (National Research Council, 1996), CLA has been subject of intensive research in the biomedical field. More than 4000 papers can be found on the ISI Web of Science (Thomson Reuters, USA) with "conjugated linoleic acid" as keyword (August 2009). In 1985, Pariza and Hargrave reported for the first time that an extract of grilled ground beef exhibited a mutagenesis inhibitory activity. The active molecules in that extract were later identified as a mixture of conjugated isomers of linoleic acid, named CLA (Ha *et al.*, 1987). CLA has also attracted interest in the scientific community because of its potential effects on atherosclerosis prevention, improvement of immunological function, and modulation of diabetes and body composition, specifically, by reducing body fat and increasing lean masses (Wahle *et al.*, 2004). These last properties were the driving force for the begining of CLA supplements commercialisation in 1996, especially, for overweight subjects and body-builders.

CLA is a natural food component found in the lipid fraction of ruminant meat, milk and dairy products (Sehat *et al.*, 1998). Food industry considers the incorporation of CLA in some foods claiming to promote beneficial effects to consumer's health. However, if some reports describe that the consumption of CLA isomers has beneficial effects, others mention harmful effects. The main concerns over CLA use identified so far are lipodystrophy, fatty liver and insulin resistance (Wahle *et al.*, 2004). Discrepancies found in animal and human trials denote how important is to continue exploiting CLA effects.

By the mid-twentieth century, dietary habits have changed, firstly in industrial regions and more recently in developing countries (WHO, 2003). Largely plant based diets have been replaced by high fat and high energy diets with considerable ingestion of animal based foods (WHO, 2003). Yet, fat intake is essential for a healthful diet, and the total amount and the type of fat consumed are key factors that should be balanced. Actually, the World Health Organization (WHO) recommends a fat intake of 15-30% (of total diet energy) of which less than 10% are saturated, 6-10% are polyunsaturated and less than 1% are *trans* fatty acids (WHO, 2003). The so-called Western diet, widely diffused in Europe and North America, provides more than 35% of calories and contains excessive saturated and *trans* fatty acids, as

well as, high cholesterol levels (Cordain *et al.*, 2005). There is evidence that high fat diets enriched in saturated fat increase the risk of coronary heart disease (Hu *et al.*, 2001) and may directly increase the prevalence of obesity (Li *et al.*, 2008). Together with the improvement of social conditions and sedentary lifestyles, the wrong nutritional habits are highly correlated with increased rates of obesity and related disorders (WHO, 2000).

Overweight and obesity states result from an abnormal excessive fat accumulation, and may be characterised by the body mass index (BMI): weight (in kilograms) divided by the square of the height (in metres). As defined by the WHO (2000), if BMI value is between 25 and 30, the person is considered overweight, and if greater than 30, the person is considered obese. More than half of the Portuguese population (53.6%), aging from 18 to 64 years old, is overweight or obese, and has an increased cardiovascular health risk associated with high waist circumference (Do Carmo *et al.*, 2008). The dysregulation of adipose tissue, in particular on the abdominal region, plays a critical role on insulin resistance and atherogenesis (Murdolo & Smith, 2006). The relation between these disorders is associated with a low-grade inflammation state of adipose tissue that results in the secretion of many molecules, adipokines and others, with metabolic, hormonal and vascular actions (Murdolo & Smith, 2006).

Facing the worldwide health problems related to obesity, scientific research is focused on discovering new compounds that could attenuate and even prevent such scenario. Regarding the potential CLA isomers properties in many areas, such as body composition, glucose and lipid metabolisms, and inflammatory system, efforts have been conducted to clarify its effects and provide new insights about their molecular action. The present thesis comprises the investigation for CLA effects in combination with saturated fat based diets, using two distinct animal models, normal-weight and genetically obese rats.

This thesis is structured in 8 chapters. The Chapter 1, “Scientific background and objectives”, introduces relevant concepts about fatty acids and their metabolism, obesity and related disorders. This chapter also describes fundamental data about CLA, as well as, a short state-of-art of its mechanisms. The objectives of this work are described at the end of this first section. The chapters 2 to 7 are based on scientific manuscripts, already published (4) or submitted (2), to international peer reviewed journals. Like the manuscripts, each chapter is composed by an abstract, introduction, description of experimental procedures, results, discussion and conclusions.

The chapter 2 presents the CLA isomeric profile of the most consumed brands of CLA-rich products in Portugal. Based on this isomeric profile and on national statistics of consumption habits, the chapter also presents the estimation of average daily intake of CLA by the Portuguese population.

Chapters 3 and 4 describe the most relevant data obtained from one experimental trial with Wistar rats in which the biological effects of *c*9,*t*11 and *t*10,*c*12 CLA isomers, individually or combined, were tested. To evaluate possible anti-adipogenic effects of CLA, the morphology of adipocytes from two different fat depots was assessed by histometry, in chapter 3. Chapter 4 reports variations on the membrane permeability of kidney proximal tubules to water and glycerol fluxes.

Chapters 5, 6 and 7 enclose several results from a trial with obese Zucker rats that were fed with CLA and saturated fats from vegetable and animal origin. Chapter 5 describes the fatty acid profile of liver and muscle, in order to further understand the CLA effects on fatty acid metabolism and its incorporation in different tissues. The metabolic state of the animals assessed by serum biochemical parameters and adipokines levels is discussed in chapter 6. Additionally, it was also investigated the relationship between fatty acids from adipose tissue and the adipokines serum profile. Chapter 7 describes how dietary treatments may influence cell death by apoptosis on adipocytes and hepatocytes exploiting possible related molecular pathways.

Finally, chapter 8 intends to summarise and discuss in an integrated form the results obtained in each of the six previous chapters, the main conclusions and revelant perspectives for future research in this topic.

CHAPTER 1 Scientific background and objectives

1.1. DIETARY FATTY ACIDS AND LIPID METABOLISM

In human diet, the presence of lipids is essential for a healthy growth and organism homeostasis. Lipids have a primarily role as an energy source and on membrane lipid composition but many other biological functions are well established: regulation of thermogenesis, synthesis of biologically active molecules and regulation of genes associated with glucose and lipid metabolisms (Roche, 2005).

The majority of dietary fatty acids are consumed as acyl esters, usually triacylglycerols (a glycerol molecule esterified with three fatty acids), and to a lesser extent as free fatty acids and phospholipids (Chow, 2000). Fatty acids are classified as saturated or unsaturated depending on the presence of double bonds in their structures. Fats from vegetable origin contain more polyunsaturated fatty acids (PUFA) than those from animal origin, which contain more saturated fatty acids (SFA), as well as, cholesterol. The excessive ingestion of SFA has been associated with the development of several important diseases including coronary artery disease, obesity, diabetes and inumerous cancers (Hu *et al.*, 2001). Regarding monounsaturated fatty acids (MUFA), oleic acid (18:1*cis*9, 18:1*c*9) is highly consumed in the Mediterranean diet and is linked to a protective effect on cancer and cardiovascular diseases (Escrich *et al.*, 2007). The polyunsaturated linoleic (18:2*n*-6) and α-linolenic (18:3*n*-3) fatty acids are considered essentials because it is not possible to synthesise them endogenously. Through the conversion of desaturase and elongase enzymes, linoleic and α-linolenic acids are converted to arachidonic (20:4*n*-6) and docosahexaenoic (22:6*n*-3) acids, respectively (Figure 1.1). Both *n*-6 and *n*-3 PUFA series synthetise eicosanoids that exert a complex control over many body systems and as messengers in the central nervous system (Calder, 2006). There is a link between fatty acids and inflammation due to the *n*-6 eicosanoids family of inflammatory mediators generated from 20 carbon PUFA, as arachidonic acid, liberated from cell membrane phospholipids (Calder, 2006). Increased consumption of the long chain *n*-3 PUFA, as eicosapentaenoic (20:5*n*-3) and docosahexaenoic acids, typically found in oily fish, decreases the amount of arachidonic acid in cell membranes diminishing arachidonic acid-derived eicosanoids (Healy *et al.*, 2000).

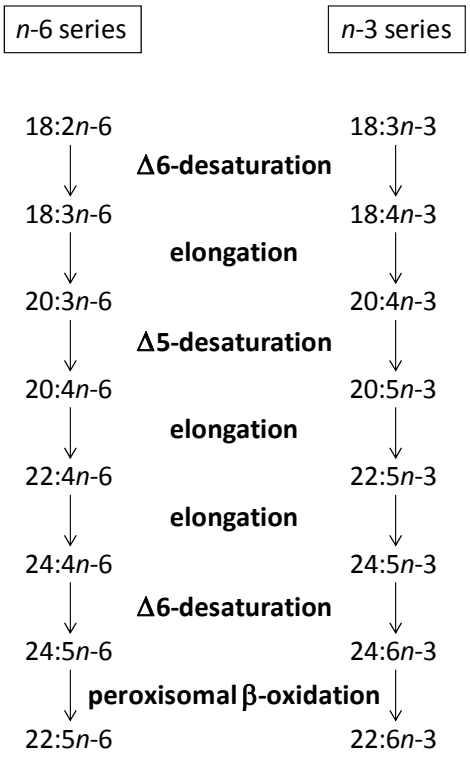

Figure 1.1 Metabolic pathways of linoleic and α-linolenic acids.

1.1.1. Digestion, absorption and transport of fatty acids and triacylglycerols

Dietary fatty acids face a complex process of digestion and subsequent absorption with multiple steps due to the insolubility of lipids in aqueous media. Triacylglycerols hydrolysation to fatty acids and monoacylglycerols is essential before absorption. The activity of lipases begins in the mouth and continues in the stomach hydrolysing primarily short and medium chain fatty acid linkages to triacylglycerols. Very short chain (up to 10 carbons) fatty acids are absorbed directly from the stomach into the venous circulation as free fatty acids. The other fatty acids passed into the intestinal lumen, where the pancreatic lipase (EC 3.1.1.3) hydrolyses fatty acids from positions 1 and 3 to yield 2-monoacylglycerols (Tso, 1985). The presence of bile acids is also required for the intestinal absorption of fatty acids and free cholesterol by promoting emulsification and solubilisation (Danielsson, 1963). Fatty acids and monoacylglycerols enter in the intestinal cell by simple diffusion into the lipid membrane or by transmembrane carrier proteins (Ryan & Van der Horst, 2000). In the intestinal cell, fatty acids are re-esterified for further metabolism.

Except for free fatty acids, which circulate bound to serum albumin, the transport of triacylglycerols and cholesterol in the blood stream requires the formation of stable structures in aqueous media, known as lipoproteins. The largest lipoproteins are the chylomicrons and

are synthesised by the intestine to transport triacylglycerols and cholesterol from the intestinal epithelium to peripheral tissues (Mahley *et al.*, 1984). The very low density lipoproteins (VLDL) are mainly secreted by the liver, and transport mainly the triacylglycerols endogenously formed (Mahley *et al.*, 1984). Following secretion from intestinal cells or liver, chylomicrons and VLDL move through the capillary blood stream, and its triacylglycerols are hydrolysed by the lipoprotein lipase (LPL, EC 3.1.1.34). LPL is attached to the interior walls of the capillary blood vessels, being specially active in the adipose tissue, lactating mammary gland, heart and skeletal muscle (Braun & Severson, 1992). The fatty acids released diffuse into the cells to be used as energy source or stored as triacylglycerols. After triacylglycerol hydrolysis, the resultant lipoprotein particles are termed chylomicrons remnants and intermediate-density lipoproteins (IDL). Remnants and IDL are actively removed by the liver but part of IDL are converted to low density lipoproteins (LDL), which are end products of the intravascular VLDL metabolism (Hussain *et al.*, 1996). LDL primarily transports cholesterol esters to the peripheral tissues, where they are hydrolysed to free cholesterol and then reacylated (Sodhi *et al.*, 1978). In an excess calories ingestion scenario, the liver catabolises chylomicron remnants, synthesises triacylglycerols and forms VLDL that are released in the circulation (Havel, 1985). The smallest lipoproteins are high density lipoproteins (HDL), and appear to arise from several sources, including liver and intestine (Eisenberg, 1984). HDL have an important anti-atherogenic function by mediating the reverse transport of cholesterol. Particles of HDL acquire the excess free cholesterol from non-hepatic cells, convert it to cholesterol esters and transport them to the liver and steroidogenic organs, in which they are used for the synthesis of lipoproteins, bile acids, vitamin D and steroid hormones (Von Eckardstein *et al.*, 2001).

Besides fatty acids, triacylglycerols are composed by glycerol, an important intermediate molecule in glucose and lipid metabolisms. Glycerol is a water-soluble molecule that is released from its storage within triacylglycerols during lipolysis. The molecular mechanism involved in the transport of glycerol from adipocytes remains not very clear. Yet, it is known that some aquaporins (AQP) promote a protein channel mediated transport and are able to permeabilise glycerol, additionally to water (Maeda *et al.*, 2004). Glycerol is an important substrate for glucose synthesis and, during fast, 40-100% of circulating glycerol is converted to glucose (Bortz, 1972). A reduction in membrane permeability to glycerol leads to an increase concentration of glycerol inside the cell. Activation of glycerol kinase (EC 2.7.1.30), the enzyme that catalyses the conversion of glycerol to glycerol-3-phosphate, favours the progressive accumulation of triacylglycerols (Frühbeck, 2005). The liver is responsible for the

major glycerol uptake with the remainder metabolised by other tissues (Gidez & Karnovsky, 1954). Besides the liver, kidney plays a major role in mammalian glycerol metabolism, accounting for up to 20% of the whole-body glycerol turnover (Gidez & Karnovsky, 1954).

1.1.2. Metabolism of fatty acids and triacylglycerols

Depending on the food energy supply, the fatty acids are mobilised in different organs through different pathways (Figure 1.2). The liver plays a central role in the lipid metabolism through uptake of fatty acids, *de novo* fatty acid synthesis, fatty acid oxidation, and assembly and secretion of VLDL (Den Boer *et al.*, 2004). In the adipose tissue, fat storage is determined by the balance between fat synthesis (lipogenesis) and fat oxidation (lipolysis and fatty acid oxidation).

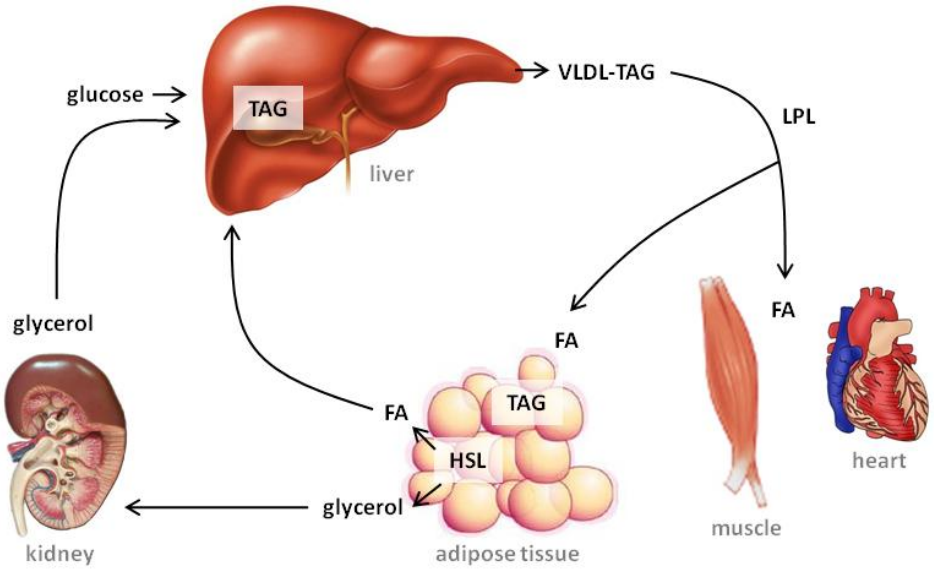

Figure 1.2 Different fates of triacylglycerols (TAG) from liver (adapted from Den Boer *et al.*, 2004). FA, fatty acids; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; VLDL, very low density lipoprotein.

- ***Lipogenesis***

Lipogenesis encompasses the process of *de novo* fatty acid synthesis, elongation/desaturation of fatty acids and subsequent triacylglycerol synthesis. In humans, the liver is the major organ of lipogenesis, while in rodents both liver and adipose tissue are important lipogenic sites (Hillgartner *et al.*, 1995). *De novo* fatty acid synthesis occurs in the cytosol and is a sequential process in which acetyl (2-carbon) units are added successively to an initial molecule, usually acetyl-CoA, derived from glucose metabolism. In the first step of fatty acid synthesis, acetyl-CoA is converted to malonyl-CoA, reaction that is catalysed by the acetyl-CoA carboxylase

(ACC, EC 6.4.1.2) enzyme (Hillgartner *et al.*, 1995). Further conversion of malonyl-CoA to saturated long chain fatty acids is mediated by fatty acid synthase (FAS, EC 2.3.1.85), a complex polypeptide containing seven distinct domains with diferent catalic activities necessary to elongate a growing fatty acid (Smith, 1994).

In animal tissues, a 16-carbon fatty acid (16:0, palmitic acid) is the predominant product of *de novo* fatty acid synthesis pathway (Volpe & Vagelos, 1973). Palmitic acid may undergo desaturation and/or elongation reactions to form other fatty acids. In the liver or adipose tissue, these newly formed fatty acids are esterified to glycerol-3-phosphate to form triacylglycerols. Considerable amounts of stearic (18:0) and oleic acids are present in adipocytes by palmitic acid conversion. In the microsomes, fatty acid elongase acts on stearic acid adding a malonyl-CoA molecule. The concentration of stearic acid in the tissues is regulated by the presence of stearoyl-CoA desaturase (SCD, EC 1.14.19.1) which converts stearic acid to oleic acid. The SCD enzyme, also known as Δ9-desaturase, is responsible for introducing a *cis* double bond at the Δ9 position (Ntambi, 1999). Playing with the ratio saturated versus monounsaturated fatty acids, the SCD has an important function in lipid fluidity regulation and cell-cell interaction (Ntambi, 1999). Diets rich in linoleic acid diminished SCD activity in Wistar rats (Jeffcoat & James, 1978).

Most fatty acids are esterified to form triacylglycerols, a form of energy storage in adipose tissue. Glycerol molecules are taken up by cells and used for glucose synthesis or to re-form triacylglycerols. In the liver and many other tissues, most fatty acids are esterified to form phospholipids important constituents of intracellular and plasma membranes. The liver actively synthesises triacylglycerols in the presence of high concentrations of free fatty acids from the blood and export them into VLDL. The esterification of fatty acids in adipose tissue increases with increasing energy intake and is lower in periods of dietary energy deficit. In abundant dietary energy situations, the high levels of insulin secreted increases lipid synthesis in liver and adipose tissue (Bazin & Lavau, 1982). Hyperinsulinaemia induced in rats increased glucose transporter-4 (GLUT-4) protein and mRNA expressions, as well as, FAS and ACC enzyme activities in the adipose tissue (Assimacopoulos-Jeannet *et al.*, 1995). In contrast, glucagon and catecholamines inhibit FAS activity. In this manner, fat synthesis is inhibited when mobilisation of energy stores is required (Hillgartner *et al.*, 1995).

Many studies have revealed that dietary fatty acids are able to modulate the gene expression of lipogenic enzymes. Dietary linoleic, α-linolenic and arachidonic acids exert a decrease in

FAS activity and in the rate of fatty acid synthesis in rat liver (Clarke *et al.*, 1976). The levels of expression of genes encoding ACC, FAS, GLUT-4 and SCD are decreased (60-90%) by dietary *n*-3 and *n*-6 PUFA, in the liver and adipose tissue (reviewed by Sessler & Ntambi, 1998). Furthermore, many reports showed specific effects depending on the fat depot. The gene expression of FAS and LPL in retroperitoneal adipose tissue were affected by *n*-3 PUFA, contrarily to non-response subcutaneous and inguinal depots (Raclot *et al.*, 1997).

- ***Lipolysis***

During a negative energy balance, the mobilisation of triacylglycerols from adipose tissue occurs by hydrolysation into free fatty acids and glycerol, a biological phenomenon known as lipolysis. The initial step of lypolysis is catalysed by hormone-sensitive lipase (HSL, EC 3.1.1.3) (Yeaman, 1990). The free fatty acids released, in the absence of stimuli to re-esterify, diffuse from the cell into the blood. There, they are adsorbed by serum albumin and circulate reaching various tissues as a complex of fatty acid-albumin. The physiological condition of the organism coordinates the balance between lipolysis and lipogenesis, regulating adipose tissue to accrete or release energy stores. Insulin stimulates uptake of glucose and glycolysis (metabolic pathway for glucose degradation), increasing the glycerol-3-phosphate available for lipogenesis (Ducluzeau *et al.*, 2002). In opposition, when blood glucose levels are low, glucagon signals adipocytes to activate HSL, and to convert triacylglycerols into free fatty acids. Dietary fatty acid composition may influence hormone-sensitive lipolysis in adipose tissue, as SFA that exert an inhibitory effect influencing several points in the lipolytic cascade (Awad & Chattopadhyay, 1986). Linoleic acid decreases the level of lipolysis in adipose tissue through the prostaglandins production (Larking & Nye, 1975), which are molecules with an antilipolytic action (Van Dorp *et al.*, 1964).

- ***Fatty acid oxidation***

In hepatocytes, the oxidation of fatty acids, or β-oxidation, is an important source of energy for adenosyl-triphosphate (ATP) production in the mitochondria. Free fatty acids are activated by fatty acyl-CoA synthetase (E.C. 6.2.1.3.), found in microsomes and outer mitochondrial membrane, to form fatty acyl-CoA. The fatty acyl-CoA may either enter the mitochondria for oxidation or be esterified within the endoplasmic reticulum. The degree of fatty acid oxidation by the liver is regulated by the supply of fatty acids to the liver via lipolysis and by the

partition between mitochondrial oxidation and microssomal esterification (Mannaerts *et al*., 1979).

Short (up to 12 carbons) and medium chain (between 12 and 16 carbons) fatty acids diffuse into mitochondria to be oxidised but long chain fatty acids (more than 18 carbons) must be transported (Schrauwen *et al*., 2003). Therefore, the entry of these long chain fatty acids into the mitochondria is regulated by the activity of the carnitine palmitoyltransferase I (CPT-I, EC 2.3.1.21) enzyme. CPT-I catalyses the formation of fatty acyl-carnitine from fatty acyl-CoA and free L-carnitine. The fatty acyl-carnitine is transported across the mitochondrial membrane by a specific carrier protein and is reconverted to acyl-CoA within the mithocondrial matrix by CPT-II action. The activity of CPT-I is inhibited by the interaction with malonyl-CoA (McGarry & Brown, 1997), the product of the first step of lipogenesis catalysed by ACC. Facing a negative energy balance, the lower levels of insulin result in decreased concentrations of malonyl-CoA and increased rates of fatty acid oxidation (Hamel *et al.*, 2001). The mitochondrial oxidation of fatty acyl-CoA forms acetyl-CoA that enters into the Krebs cycle to produce energy. Alternatively, acetyl-CoA participates in ketone bodies formation. Under conditions of increased non-esterified fatty acid uptake, as during starvation, the liver often produces large amounts of the ketone bodies in the process known as ketogenesis (McGarry & Foster, 1980).

For very long chain fatty acids (22 carbons or more) the β-oxidation is more active in peroxisomes than in mitochondria. In the rat model, conditions leading to increased supply of fatty acids in the liver, such as high dietary fat, starvation and diabetes, induce peroxisomal β-oxidation enzymes, which denotes the important role of this pathway helping with increased flux of fatty acids (Singh, 1997).

1.2. OBESITY AND RELATED METABOLIC DISORDERS

Obesity can be defined as an excess of body fat (BMI>30 in humans) and this abnormal adipose tissue deposition may have significant consequences to the subject. Conventionally, white adipose tissue was seen as a tissue that can store fatty acids as triacylglycerols in times of positive energy balance, and release the energy from free fatty acids and glycerol in times of energy need (Murdolo & Smith, 2006). However, adipose tissue is not an homogeneous organ, consisting in a variety of different cell types, as adipocytes, preadipocytes, stromal/vascular cells and macrophages (Fain, 2006). It is now known that adipose tissue is

capable of synthesising and releasing a variety of hormones and cytokines, called adipokines. These are implicated in a wide range of pathophysiological processes, including, appetite and energy balance, glucose homeostasis, lipid metabolism, blood pressure regulation and angiogenesis (Kershaw & Flier, 2004). In the past two decades, adipose tissue was recognised as a central player in the subclinical inflammatory state characteristic in patients with obesity and insulin resistance (Van Gaal *et al.*, 2006). Excessive adipose tissue energy storage results in increased fatty acid flux to other tissues and increased triacylglycerol storage in peripheral tissues, which promotes inflammation, insulin resistance and endothelial dysfunction (Figure 1.3).

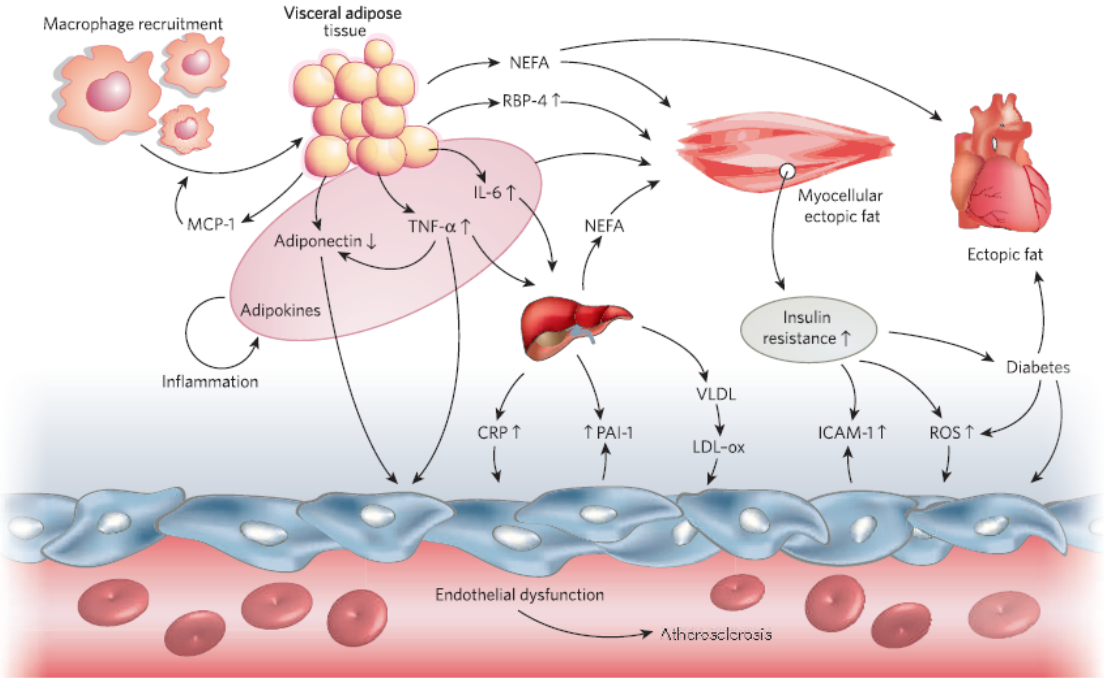

Figure 1.3 Relationship among visceral adipose tissue, inflammation, insulin resistance and endothelial dysfunction (from Van Gaal *et al.*, 2006). MCP-1, monocyte chemoattractant protein-1; NEFA, non-esterified fatty acid; RBP-4, retinol binding protein-4; IL-6, interleukine-6; TNF-α, tumor necrosis factor-alpha; CRP, C-reactive protein; PAI-1, plasminogen activator inhibitor-1 ; VLDL, very low density lipoprotein; LDL-ox, oxidised low density lipoprotein; ICAM-1, intercellular adhesion molecule-1; ROS, reactive oxygen species.

Except for adiponectin, the serum levels of adipokines are elevated in obesity, as for leptin, tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor-1 (PAI-1) and several interleukines (IL). Interventions that reduce body fat mass may also lower adipokines levels (Vendrell *et al.*, 2004). Adiponectin is specifically produced by the adipose tissue, regulates lipids and glucose metabolisms and possesses anti-inflammatory properties (Kadowaki & Yamauchi, 2005). Adiponectin is able to improve insulin sensitivity and reduce the hepatic triacylglycerol content (Yamauchi *et al.*, 2001). As shown in Figure 1.3, several adipokines enhance the attachment and migration of monocytes into the vessel wall, and their conversion

into macrophages, where they phagocytose oxidised LDL and form lipid-lader foam cells (Van Gaal *et al*., 2006). IL-6 has pro-inflammatory activity itself and through increasing the levels of TNF-α and IL-1β, and also stimulates liver production of C-reactive protein (CRP), all implicated in atherogenesis (Lyon *et al*., 2003). PAI-1 is an acute-phase protein mainly produced by the liver but also by the adipose tissue. It is a regulatory protein of the coagulation cascade and act as a promoter of atherogenesis, increasing deposition of platelets and fibrinous products to developing plaques (Berg & Scherer, 2005). One important factor produced by the adipocytes underlying macrophages infiltration adipose tissue is the monocyte chemoattractant protein-1 (MCP-1) (Murdolo & Smith, 2006). Leptin is another relevant adipokine. Because it is secreted by adipose cells in proportion to body fat store, leptin has the function to signal the hypothalamus reducing the appetite and the insulin secretion (Frühbeck *et al*., 2001). Since the discovery of leptin, several other hormones and cytokines secreted by adipocytes and cells of the gastrointestinal system have been characterised, as adiponectin and ghrelin. Ghrelin is the key appetite hormone and it is highly produced by an empty stomach but is decreased with feeding (Depoortere, 2009).

In humans, the cellular composition of adipose tissue can vary substantially depending on anatomical location and body weight (Fain, 2006). Additionally, expression of adipokines seems to be dependent on adipose depot. Visceral fat appears to produce several of these adipokines more actively than subcutaneous depot (Lyon *et al.*, 2003). In fact, human visceral and subcutaneous fat depots exhibit alterations in the expression of specific genes that contribute to pro-inflammatory and insulin resistance states (Dolinková *et al.*, 2008).

1.2.1. Insulin resistance and type 2 diabetes

There is a strong link between obesity and diabetes due to the adverse effects of excess body fat on the insulin response with impairment of its action, which leads to a compensatory insulin resistance (Saltiel, 2000; Roche, 2005). Insulin is the principal hormone responsible for glucose homeostasis and for the stimulation of glucose transport (Shepherd & Kahn, 1999). The secretion of insulin by the pancreas is controlled by glucose concentrations in the bloodstream and rapidly reaches its target tissues (mainly liver, muscles and adipose tissue), where it interacts with its receptor (Taha & Klip, 1999). Most cells in the human body possess insulin receptors to activate the absorption of glucose from the bloodstream (Joost, 1995). On the plasma membrane, insulin receptors activate the expression of glucose transporters: GLUT-4 in muscle and adipose tissue, and GLUT-2 in liver (Mueckler, 1994). Insulin

resistance is a pathological state in which target tissues fail to respond properly to normal levels of circulating insulin (Reaven, 1988). In prediabetic insulin resistant individuals, the pancreatic β-cells compensate the peripheral insulin resistance, producing excessive insulin quantities to maintain normal glucose levels. With continuous impairment of insulin sensitivity, the pancreas fails to compensate and progressive hyperglycaemia develops in type 2 diabetes (Weyer *et al.*, 1999). A variety of cellular and molecular anormalies are behind insulin resistance, as dysfunctional insulin receptors, anomalous receptor signalling pathways and abnormalities in glucose transport or glucose metabolism (Reaven, 1988). The dyslipidaemia seen in many insulin resistant conditions is often associated with high levels of triacylglycerols and low levels of HDL-cholesterol, in addition to increased levels of free fatty acids (Arner, 2002). In obese patients with type 2 diabetes, the adipose tissue release more free fatty acids than in lean subjects and these free fatty acids may impair insulin action and glucose metabolism (Arner, 2002). Evidences suggest that metabolic stressors (as free fatty acids) and pro-inflammatory signals (as TNF-α) induce insulin resistance by inhibiting insulin signalling (Zick, 2001).

- ***Dietary fatty acids and insulin resistance***

Molecular studies have demonstrated that fatty acids effects on glucose and lipid metabolisms are due to their ability to activate specific nuclear receptors that cause peroxisomal proliferation (Khan & Heuvel, 2003). These receptors, peroxisome proliferators-activated receptors (PPAR), bind to specific peroxisome proliferators response elements that are located in the regulatory region of genes associated with lipid metabolism (Khan & Heuvel, 2003). The subtype PPAR-α (the predominant form of PPAR in the liver) is recognised as regulator of fatty acid oxidation in the liver (Delerive *et al.*, 2001). PPAR-γ is the nuclear receptor that controls lipid metabolism in adipose tissue, and regulates adipocyte differentiation, proliferation, and lipogenesis (Gregoire, 2001). In rodents, the activation of PPAR-α stimulated the fatty acid oxidation with consequent reduction of adipose tissue depots, decrease in ectopic lipid storage in muscle and liver and the improvement of insulin sensitivity in these tissues (Haluzík & Haluzík, 2006). Other fatty acid-mediated regulators of lipogenic genes have been studied, as sterol regulatory element-binding proteins (SREBP)-1 (Brown & Goldstein, 1997). SREBP are a family of membrane-bound transcription factors that activate genes involved in the synthesis of cholesterol and fatty acids and their uptake from plasma lipoproteins (Brown & Goldstein, 1999). The ingestion of *n*-3 and *n*-6 PUFA in place of SFA changes the pattern of lipid metabolism in the liver by increasing lipolitic genes.

Possible mechanisms are the PPAR activation by PUFA and the conversion of PUFA to eicosanoids (Jump & Clarke, 1999).

It has been suggested that consumption of MUFA decreases blood triacylglycerols by increasing fatty acid β-oxidation, through activation of PPAR-α, or by reducing the activation of SREBP-1 and inhibiting lipogenesis (Soriguer *et al.*, 2006). Additionally, the replacement of carbohydrate and saturated fat with MUFA led to a reduction in glucose and blood pressure, and to an increase in HDL-cholesterol in patients with type 2 diabetes (Julius, 2003). A relationship between plasma fatty acid composition and insulin resistance have been previously reported. Men, who after 4 years, developed impaired fasting glycaemia or type 2 diabetes presented increased SFA and decreased PUFA in serum (Laaksonen *et al.*, 2002). An epidemiological study also showed that a higher intake of SFA and a low PUFA/SFA ratio are related to increased cardiovascular diseases risk among women with type 2 diabetes (Tanasescu *et al.*, 2004). The insulin action was improved by arachidonic, eicosapentaenoic and docosahexaenoic acids, which enhanced the membrane fluidity and the number of insulin receptors (Simopoulos, 1994).

1.2.2. Non-alcoholic fatty liver disease

In parallel with obesity and type 2 diabetes, the prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing in developed countries (Angulo, 2002). NAFLD is a clinicopathological syndrome with a wide spectrum of liver damage ranging from steatosis, to steatohepatitis, and ultimately, to cirrhosis (Feldstein & Gores, 2005). Hepatic steatosis is characterised by the presence of significant amounts of triacylglycerols within hepatocytes and may be a direct consequence of: increased delivery and *de novo* synthesis of free fatty acids in the liver, decreased β-oxidation, and decreased synthesis and secretion of VLDL (Browning & Horton, 2004). Excessive accumulation of triacylglycerols in the liver is strongly related with peripheral insulin resistance that affects glucose and lipid metabolisms (Angulo, 2002). Insulin resistance inhibits glucose uptake via GLUT-4 in both skeletal muscle and adipose tissue, redirecting glucose to the liver. Moreover, lipogenesis is stimulated by insulin, rising the hepatic triacylglycerol content (Postic & Girard, 2008). The prevalence of other metabolic abnormalities associated with insulin resistance, such as, impairment of glucose tolerance, hypertriglyceridaemia, and decreased levels of HDL-cholesterol are concomitant exacerbated in NAFLD subjects (Tsai *et al.*, 2008).

- ***NAFLD and apoptosis***

In vivo and *in vitro* models of NAFLD provided many evidences that fatty acid accumulation results in elevated cell death by apoptosis (Feldstein & Gores, 2005). Apoptosis, also termed as programmed cell death, is an energy-dependent process of cellular deconstruction that contrasts morphologically with necrosis (Schwartzman & Cidlowski, 1993). Apoptosis is a form of cell death that occurs during several physiological and pathological situations in multicellular organisms, and constitutes a common mechanism of cell replacement, tissue remodelling and removal of damaged cells (Renehan *et al.*, 2001). As a complex process, apoptosis is defined by a series of biochemical and morphological modifications, including condensation of chromatin, shrinkage of cytoplasm and nuclear compartments, degradation of DNA into oligonucleosome-length fragments and compartmentalisation of nuclear material into vesicular apoptotic bodies (Kerr *et al.*, 1972). Apoptosis occurs by several molecular pathways that may interconnect to each other. Studies using a genetic model of NAFLD, the obese mouse *ob*/*ob*, have shown increased expression of specific mediators of both extrinsic and intrinsic pathways of apoptosis (Rashid *et al.*, 1999). Two fundamental pathways may execute apoptosis: one is mediated by death receptors on cellular surface (the extrinsic pathway) and the other is organelle based (the intrinsic pathway).

The extrinsic pathway is iniated by cell death receptors, as Fas, TNF-α receptor 1 (TNF-R1) and TNF inducing ligand receptor (TRAIL-R). These receptors trigger intracellular cascades that activate death-inducing proteolytic enzymes, especially caspases (Yoon & Gores, 2002). In particular, Fas is a cell surface glycoprotein belonging to the tumor necrosis factor family (Itoh *et al.*, 1991). The Fas receptor bond induces apoptosis by promoting proteolytic cleavage of intracellular caspases (Scaffidi *et al.*, 1998). Caspases are a family of intracellular cysteine peptidases that play a pivotal role in the initiation and execution of apoptosis induced by various stimuli (Green, 2000).

In the intrinsic pathway, apoptosis can be initiated by several intracellular organelles triggering lysosomal permeabilisation, nuclear DNA damage, endoplasmic reticulum (ER) stress and mitochondrial dysfunction. The ER is a dynamic organelle, serving as both a specialised compartment for the folding and assembly of secretory proteins and as a sensor for certain types of cellular stress (Rutkowski & Kaufman, 2004; Kim *et al.*, 2008). Although ER stress is better described as an intrinsic, rather than extrinsic apoptotic signal, apoptosis induced by ER stress appears to rely on elements of both pathways, and thus the relative

importance of either of these pathways is unclear (Rutkowski & Kaufman, 2004). The ER stress may lead also to c-Jun NH_2-terminal kinase (JNK) stress activation, a protein kinase that phosphorilates c-Jun protein, initiating a pro-apoptotic phosphorilation cascades (Urano *et al.*, 2000; Özcan *et al.*, 2004). CHOP (CCAAT/enhancer-binding protein homologous protein) is a transcription factor implicated in programmed cell death, and is regulated by the ER chaperone binding protein (BiP) (Zinszner *et al.*, 1998). In turn, the ER chaperone BiP is a known target of the activating transcription factor (ATF)-6α, too. ATF-6α is an ER transmembrane protein that under normal conditions is retained in the ER through interaction with BiP. Mitochondrial dysfunction results in release of several pro-apoptotic proteins into the cytosol including cytochrome c, which then forms an activation complex with apoptotic-protein activation factor-1 (Apaf-1) and caspase 9. This complex activates the downstream effectors caspases 3, 6 and 7, culminating in the apoptotic demise of the cell (Li *et al.*, 1997).

1.3. POTENTIAL EFFECTS OF CONJUGATED LINOLEIC ACID ON BODY FAT COMPOSITION, OBESITY AND INSULIN RESISTANCE

The term CLA describes a mixture of geometrical and positional isomers derived from linoleic acid, in which only one single bond separates the two double bonds (Figure 1.4). CLA is formed when either one or two double bonds present in linoleic acid shift resulting in two conjugated double bonds.

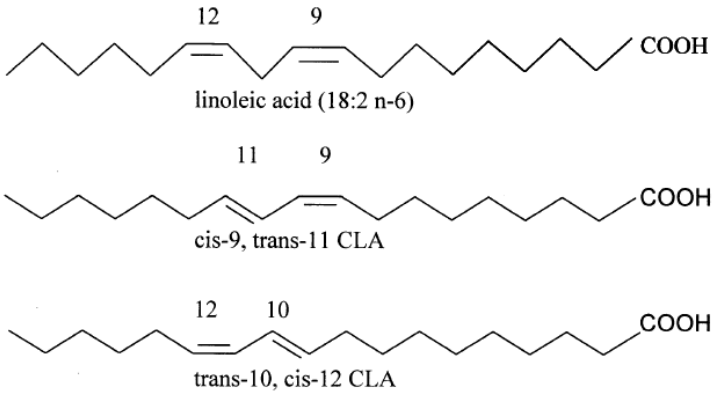

Figure 1.4 Structures of linoleic acid, *c*9,*t*11and *t*10,*c*12 CLA isomers (from Evans *et al.*, 2002).

Theoretically, 28 CLA isomers, depending on the variation from carbons 6,8- to 12,14- and geometrically, can be found with configuration *trans,trans*, *trans,cis*, *cis,trans* and *cis,cis* (*t,t*, *t,c*, *c,t* and *c,c*) (Wahle *et al.*, 2004). Unless specified, CLA or CLA mixture indicates a mixture predominantly of 18:2*cis*9,*trans*11 (*c*9,*t*11) and 18:2*trans*10,*cis*12 (*t*10,*c*12) CLA

isomers. CLA isomers are naturally formed by biosynthesis in ruminant animals, using two different pathways (Figure 1.5), which mainly lead to the formation of *c*9,*t*11 isomer. A first pathway is the biohydrogenation of ingested dietary PUFA, *e.g.* linoleic acid, into stearic acid by enzymes of bacterial flora present in the rumen, such as, *Butyrivibrio fibrisolvens* (Griinari & Bauman, 1999). During biohydrogenation process, *c*9,*t*11 CLA isomer, also known as rumenic acid, and vaccenic acid (18:1*trans*11, 18:1*t*11) are formed. The finally reduction of 18:1*t*11 into 18:0 seems to be the rate limiting reaction. Hence, the intermediate products *c*9,*t*11 CLA isomer and 18:1*t*11 are accumulated, absorbed in the intestine and incorporated into different tissues (Bauman *et al.*, 1999). In a second pathway, CLA is formed endogenously from vaccenic acid through Δ9-desaturation by the activity of SCD enzyme in adipose tissue and in mammary gland of lactating cows (Corl *et al.*, 2001). The endogenous synthesis that occurs in the mammary gland of lactating cows contributes to more than 60% of CLA in the milk fat (Palmquist *et al.*, 2004). Apart from ruminant animals, the endogenous synthesis of CLA has been reported in other species, such as, mouse (Santora *et al.*, 2000), rat (Lock *et al.*, 2004), pig (Glaser *et al.*, 2002) and human (Salminen *et al.*, 1998; Adlof *et al.*, 2000; Turpeinen *et al.*, 2002). Several strains of *Bifidobacterium*, found in either adult or infant intestine, have been shown to produce CLA and, in particular, the *c*9,*t*11 isomer (Coakley *et al.*, 2006). Nevertheless, the predominant source of CLA for humans is through dietary intake (Herbel *et al.*, 1998). CLA biosynthesis in ruminants accounts for a high concentration of these isomers in meat and dairy products from these animals.

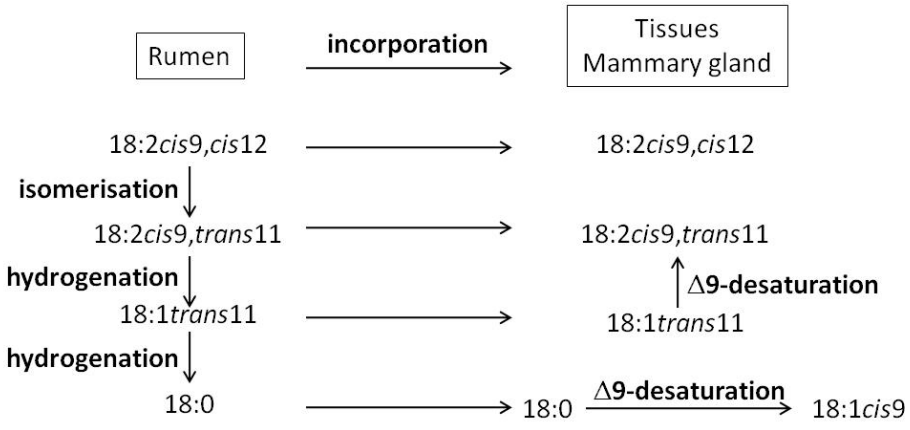

Figure 1.5 Pathways of *c*9,*t*11 CLA isomer biosynthesis.

Industrially, the production of large quantities of CLA is carried out by chemical synthesis via alkaline-catalysed isomerisation of linoleic acid, mostly using vegetable oils rich in PUFA, as corn and sunflower oils (Kellersmann *et al.*, 2006). On the contrary to CLA profile in food products, the synthesised CLA mixture presents about equal amounts of *c*9,*t*11 and *t*10,*c*12

CLA isomers, in a total content of 80-90%, and other minor CLA isomers (Christie *et al.*, 1997). As numerous biological effects of CLA are attributed to individual action of *c*9,*t*11 and of *t*10,*c*12, these two isomers are available commercially for research purposes, being purified at low-temperature crystallisation reaching more than 90% of purity (Kellersmann *et al.*, 2006). However, synthesis and isolation of single CLA isomers are more difficult and expensive than generating a mixture of CLA isomers. Therefore, the majority of research has been conducted with CLA mixtures.

Commercial sources of CLA are available in form of capsules that usually contain a mixture of CLA isomers obtained by chemical synthesis. Total CLA contents in these supplements may vary between 65-80% (Yu *et al.*, 2003). Supplements provide an additional oral source of CLA and generally are advertise to integrate a loss weight dietary regimen. CLA supplementation have been recommended of 0.8-3 g *per* day based on anticancer effects of CLA (Parish *et al*., 2003). These values were extrapolated from an experimental study where a supplementation of 0.1% CLA in the diet significantly reduced the incidence of mammary carcinogenesis in rats (Ip *et al.*, 1994). The safety of commercial CLA preparations has been evaluated in numerous human clinical trials. As mentioned earlier, typically commercial CLA preparations consist almost entirely of the two biologically active isomers, *c*9,*t*11 and *t*10,*c*12, in approximately equal amounts (i.e. about 40-45% each) (Yu *et al.*, 2003).

1.3.1. Metabolism of conjugated linoleic acid isomers

It has been shown that CLA can be metabolised like other fatty acids. One possible metabolic pathway is through Δ6 desaturation, elongation, and further Δ5 desaturation, similarly to linoleic acid, as shown in Figure 1.1. The resulting conjugated metabolites, 18:3 (octadecetrienoic acid), 20:3 (eicosatrienoic acid) and 20:4 (eicosatetraenoic acid), have been found in liver and mammary gland of rats, as well as, in the adipose tissue of human subjects (Sébédio *et al.*, 1997, 2003). On the other hand, CLA may enter on the process of fatty acid β-oxidation process, either mitochondrial or peroxisomal. Examining the peroxisomal β-oxidation products of CLA, Banni *et al.* (2001) observed the presence in rat liver of two 16:2 isomers possible resulting from the removal of a two-carbon unit. In addition, conjugated 12 and 14 carbon fatty acids, other possible products from fatty acid β-oxidation of CLA, were reported in 3T3-L1 adipocyte cell cultures (Park *et al.*, 2005). Nothwithstanding, the behaviour of *c*9,*t*11 and *t*10,*c*12 CLA isomers in the oxidation process is different. The

*t*10,*c*12 is more readily oxidised than *c*9,*t*11, probably due to the positioning of their double bonds (Martin *et al.*, 2000).

The metabolisation and conversion of CLA into conjugated fatty acid by successive desaturations and elongations steps, as described above, have direct effects on the metabolism of essential fatty acids. One example is the arachidonic acid, formed from linoleic acid in three steps, Δ6 and Δ5 desaturations and elongation (Figure 1.1). CLA may interact with the arachidonic acid metabolism by competing with linoleic acid (Banni, 2002). Several studies showed a decrease on the content of linoleic acid and their metabolites, as arachidonic acid, in the mammary and adipose tissue by CLA (Banni *et al.*, 1999), and, specifically, reduced arachidonic acid in muscle phospholipid fractions in mice (Park *et al.*, 1997). The replacement of arachidonic acid concentrations by CLA does not explain the whole extent of the reduction of arachidonic acid due to the relatively small quantities of CLA compared to the changes in arachidonic acid level in the same tissue (Park *et al.*, 2007a). In fact, it has been reported that CLA can inhibit cyclooxygenase activity, which constitutes the rate limiting enzyme for prostaglandin formation (Li *et al.*, 2005). The metabolism of both unsaturated and saturated fatty acids is influenced by the presence of CLA in the diet. Furthermore, CLA modified the membrane fatty acid composition of hepatocytes by reducing MUFA resulting from the decreased activity of the SCD (Lee *et al.*, 1998). *In vitro* and *in vivo* studies, demonstrated that *t*10,*c*12 CLA isomer decreased 16:1*c*9 and 18:1*c*9 contents suggesting an inhibition of the SCD activity (Choi *et al.*, 2001; Sébédio *et al.*, 2001).

The incorporation of CLA isomers into various biological systems, as tissues and blood plasma, after CLA administration in animals and humans, has been studied (Sugano *et al.*, 1997; Hoffmann *et al.*, 2005). Moreover, the incorporation of single CLA isomers was found to be different: *c*9,*t*11 CLA is more efficiently incorporated than *t*10,*c*12 CLA isomer (Kelley *et al.*, 2006; Martin *et al.*, 2007). Concerning the assimilation in different tissues, *c*9,*t*11 isomer was preferably accumulated in liver, serum, bone and marrow (Kramer *et al.*, 1998; Li & Warkins, 1998), whereas the *t*10,*c*12 seems to be better incorporated into spleen, muscle and heart lipids (Li & Warkins, 1998). This differential tissue incorporation of individual CLA isomers suggests either a selective uptake of a particular isomer at high isomer concentration, or a rapid metabolism, or a discrimination in the uptake at low concentration (Sébédio *et al*., 2003). Recently, Tsuzuki and Ikeda (2007) observed a similar extent of lymphatic recovery of these two isomers in rats, suggesting that geometrical and positional

isomerism of the conjugated double bounds had no influence on the absorption by the small intestine.

Furthermore, CLA isomers incorporate differentially into the various lipid classes comparing to linoleic acid. CLA and its derivatives, with the exception of conjugated 20:4, are preferably incorporated into triacylglycerols, while linoleic acid and its metabolites into phospholipids (Park *et al.*, 1999). The spatial structure of the molecule could be the reason for its incorporation behaviour. The carbon chain of CLA resembles more oleic acid molecule than linoleic acid, incorporating better into triacylglycerols than into phospholipids, as oleic acid (Sébédio *et al*., 2003). The unsaturated fatty acids present in phospholipidic membrane play an important role maintaining fluidity and influencing cellular physiologic events (Sessler & Ntambi, 1998). At least a part of the CLA properties may be attributed to its action on fatty acids metabolism changing the fatty acid profile of cell membranes.

1.3.2. Biological effects of conjugated linoleic acid isomers

Regarding obesity and body composition, several studies in animals have shown the ability of CLA to reduce adiposity and increase lean mass (Park & Pariza, 2007b). However, there are heterogeneous responses of CLA to adipose tissue modulation that may be attributed to species, breed, age and fat depots studied (Domeneghini *et al.*, 2006). Park and co-workers (1997) were the first reporting that the administration of 0.5% of CLA caused a 60% decrease in body fat and a 14% enhancement in lean body mass, after 4-5 weeks of feed growing mice. In the same species, others claimed reductions in fat deposition by CLA (Tsuboyama-Kasaoka *et al.*, 2000; Park & Pariza, 2001a; Takahashi *et al.*, 2002). In fact, mice appear to be the most effective animal model regarding the CLA anti-adipogenic effect (Pariza *et al.*, 2001). Sprague-Dawley rats fed 1.5% of CLA during 3 weeks had reduced white adipose tissue weight but the reduction was not so marked as in mice (Yamasaki *et al.*, 2003). Dietary CLA also decreased body fat accumulation in a dose-dependent manner in pigs (Ostrowska *et al.*, 2003; Corino *et al.*, 2005). Although several studies have been performed by using a mixture of isomers, others suggest that the biological active isomer with anti-obesity properties is *t*10,*c*12 CLA isomer (see review Park & Pariza, 2007b). The efficacy of CLA in humans has produced inconsistent results in both normal-weight or overweight and obese subjects (Plourde *et al.*, 2008).

- ***Mechanisms of body fat reduction by CLA***

The effect of CLA on body fat reduction has been attributed to multiple mechanisms, as 1) increase of energy expenditure; 2) modulation of lipogenesis; 3) increase fatty acid β-oxidation; 4) enhancement of preadipocytes and adipocytes apoptosis; 5) modulation of adipokines and cytokines.

It has been shown consistently with the *t*10,*c*12 isomer, but not with *c*9,*t*11, that LPL inhibition in adipocytes results in a fat uptake reduction (Lin *et al.*, 2001; Park *et al.*, 2004). Also, studies have reported that CLA inhibition of SCD enzyme contribute to reduce the fat mass (Park *et al.*, 2000; Lin *et al.*, 2004). Other lipogenic enzymes, such as ACC and FAS, were markedly decreased in female C57BL/6J mice after 1% of CLA feeding (Tsuboyama-Kasaoka *et al.*, 2000). The expression of SREBP-1, that regulates lipogenic enzymes, showed a tendency to decrease with CLA feeding (Roche *et al.*, 2002). As other dietary fatty acids, CLA isomers have some features similar to peroxisome proliferators. In fact, CLA act as a ligand and activator of PPAR-α that may explain the hepatic lipid accumulation by these fatty acids (Moya-Camarena *et al.*, 1999). The CLA effect on adipocytes may be also be linked to interaction between CLA and PPAR-γ. However, CLA has been shown to reduce PPAR-γ expression in a number of publications (Tsuboyama-Kasaoka *et al.*, 2000; Takahashi *et al.* 2002), while others found that CLA increased PPAR-γ expression (Brown *et al.*, 2001; Clement *et al.*, 2002).

Other CLA effect on lipid metabolism includes the induction of β-oxidation. Studies in rodents provided data demonstrating that CLA increased peroxisomal oxidation by enhancing the CPT-I expression in skeletal muscle (Park *et al.*, 1997; De Deckere *et al.*, 1999; Pariza *et al.*, 2001; Nagao *et al.*, 2005).

The increase of apoptosis is another physiological process that may be associated with the reduction of body fat mass induced by CLA. CLA caused apoptosis in mice adipose tissue (Miner *et al.*, 2001) and in preadipocyte cultures (Evans *et al.*, 2000). Corino *et al.* (2005) observed that 0.75% of CLA increased the number of apoptotic adipocytes in the subcutaneous fat depot of heavy pigs. One potential mechanism that leads to apoptosis may involve an increase in TNF-α levels (Tsuboyama-Kasaoka *et al.*, 2000). However, this mechanism needs further research, as others have shown that CLA simultaneously decreased serum TNF-α levels and body fat mass (Yamasaki *et al.*, 2003; Bhattacharya *et al.*, 2005).

CLA may reduce body fat by modulating adipokines and cytokines release. These factors may be directly or indirectly linked to the cellular signal transduction pathways behind biological CLA effects. CLA has been shown to reduce expression and secretion of leptin (Kang & Pariza, 2001; Rahman *et al.*, 2001; Ryder *et al.*, 2001). Reduction of leptin levels by CLA may be explained by the fact that CLA reduced the total amount of adipose tissue (Park *et al.*, 2007a). Many studies showed that CLA increased adiponectin and decreased TNF-α (Pariza *et al.*, 2000; Yang & Cook, 2003; Nagao *et al.*, 2005). Others have reported modification of interleukins by CLA, as well (Brown *et al.*, 2004; Bhattacharya *et al.*, 2005). The *t*10,*c*12 CLA isomer increases the expression and secretion of several pro-inflammatory cytokines, including IL-6 and IL-8 (Brown & McIntosh, 2003; Brown *et al.*, 2004).

- ***Effect of conjugated linoleic acid on insulin resistance***

Divergent effects of CLA on insulin resistance have been reported. Houseknecht *et al.* (1998) firstly reported that CLA induced a normalisation of impaired glucose tolerance and improved hyperinsulinaemia in diabetic rats. Henriksen *et al.* (2003) reported that CLA mixture and the *t*10,*c*12, but not the *c*9,*t*11, improved glucose tolerance in obese Zucker rats. However, experimental studies in humans and other animal models have shown that CLA can have adverse effects on glucose metabolism (Tsuboyama-Kasaoka *et al.*, 2000; Taylor & Zahradka, 2004; Poirier *et al.*, 2006). For example, the *t*10,*c*12 isomer markedly decreased the insulin sensitivity in obese men with metabolic syndrome (Risérus *et al.*, 2004). The regulation of some adipokines mediated by CLA may explain some of these effects. The reduction of circulating leptin levels after CLA supplementation has been suggested to be associated with an increase of insulin resistance (Belury *et al.*, 2003; Wendel *et al.*, 2008). Although CLA has been considered an anti-inflammatory compound, it activates NF-κB, a transcripton factor that regulates several inflammatory genes expression, such as, TNF-α, IL-6 or IL-8 (Chung *et al.*, 2005; De Roos *et al.*, 2005; Poirier *et al.*, 2005). These cytokines decrease adipocyte GLUT-4 expression, which blocks cell glucose uptake and, therefore, increases the circulating levels of insulin (Winzell *et al.*, 2006). TNF-α also directly inhibits the activity of the insulin receptor (Hotamisligil, 1995).

1.4. RESEARCH OBJECTIVES

CLA is a natural dietary compound daily ingested through the regular diet. Although some studies reported the total CLA content (or *c*9,*t*11 isomer content) on some Portuguese foods, there was a lack of knowledge about the total daily CLA intake by Portuguese population and its isomeric profile. As being so, a major goal of the present work was to estimate the daily intake of CLA by the regular diet by Portuguese population (chapter 2), which comprised the following specific aims:

a) to identify and quantify the CLA isomeric profile of the most consumed CLA-rich products distributed in Portugal (chapter 2);
b) to estimate the average daily intake of total CLA and the profile of individual isomers by the Portuguese population, based on the CLA isomeric profile of CLA-rich products and on national statistics of consumption habits (chapter 2).

Other major goal of this work was the elucidation of CLA biological effects, in combination with saturated fat diets, on two distinct *in vivo* models, Wistar (chapters 3 and 4) and obese Zucker rats (chapters 5, 6 and 7). In the first trial, *c*9,*t*11 and *t*10,*c*12 CLA isomers, individually and as a mixture, were administrated to a healthy normal-weight model (Wistar rat), in combination with palm oil. Specifically, the objectives were:

c) to evaluate the morphological changes of adipose cells size and weight of epididymal and retroperitoneal fat depots (chapter 3);
d) to study the membrane permeability of the kidney proximal tubule to water and glycerol fluxes (chapter 4).

In the second trial, a mixture of CLA isomers and two different saturated fats, palm oil and ovine fat, were tested on a genetically obese model (obese Zucker rat). The specific objectives were:

e) to investigate the differential deposition of fatty acids in the liver and muscle (chapter 5);
f) to assess the serum adipokine profile and the fatty acid composition of epididymal and retroperitoneal fat depots (chapter 6);
g) to explore the levels of cell death by apoptosis and the respective molecular pathways on adipocytes and hepatocytes (chapter 7).

CHAPTER 2 Contents of conjugated linoleic acid isomers in ruminant-derived foods and estimation of their contribution to daily intake in Portugal

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Abstract

The present study provides a detailed overview of the contents of conjugated linoleic acid (CLA) isomers in the most consumed Portuguese CLA rich foods (milk, butter, yoghurt, cheese, beef and lamb meat), by using silver ion-HPLC. In addition, the contribution of these ruminant-derived foods to the daily intake of CLA isomers was estimated based on Portuguese consumption habits. The total CLA concentration in milk and dairy products ranged from 4.00 mg/g fat in yoghurt to 7.22 mg/g fat in butter, and, regarding meats, from 4.45 mg/g fat in intensively produced beef to 11.29 mg/g fat in lamb meat. The predominant CLA isomers identified in these products were *c*9,*t*11 (59.89–79.21%) and *t*7,*c*9 (8.04–20.20%). The average estimated total CLA intake for the Portuguese population was 73.70 mg/day. Milk and cheese are probably the two products with the highest contribution to the final CLA intake, as a result of their high fat content and consumption values. The results also showed that *c*9,*t*11 and *t*7,*c*9 were the isomers most prevalent, with, respectively, 76.10 and 12.56% of the total CLA intake. Being the first detailed report on the contents of total and individual CLA isomers in Portuguese commercial ruminant-derived foods, we further discuss the implication of the results for diet characteristics and human health.

2.1. INTRODUCTION

Conjugated linoleic acid (CLA) refers to a heterogeneous group of geometrical and positional isomers of linoleic acid with conjugated double bonds. These double bonds can either be *trans* or *cis* configured, and a wide spectrum of isomers with variations in position (from 6,8 to 12,14) and geometry (*t*,*t*, *t*,*c*, *c*,*t* and *c*,*c*) has been described (Prates & Mateus, 2002; Wahle *et al.*, 2004). Twenty different CLA isomers occur naturally in food, especially in ruminant-derived fat (Sehat *et al.*, 1998). The major CLA isomer (*c*9,*t*11), as well as the usually second most prevalent isomer (*t*7,*c*9), are produced in the rumen during microbial biohydrogenation of dietary 18:2*n*-6 and in the tissues through Δ9-desaturation of the rumen-derived vaccenic acid (Griinari and Bauman, 1999). It is now accepted that the major contribution to these CLA isomers in ruminant-derived milk (Corl *et al.*, 2002) and meat (Palmquist *et al.*, 2004) is the endogenous synthesis by Δ9-desaturation. With the exception of these two isomers, the origin of all other CLA isomers is supposed to arise from ruminal biohydrogenation of dietary unsaturated C18 fatty acids, even if the metabolic pathways are not yet elucidated (Collomb *et al.*, 2004).

Many experimental studies, using laboratory animals, as well as humans and cell culture systems, suggest that CLA exhibits interesting biological activities: anticarcinogenic, anti-adipogenic, antidiabetogenic, anti-atherogenic and anti-inflammatory (Wahle *et al.*, 2004). The National Academy of Sciences of the USA recognised CLA as the only fatty acid that unequivocally inhibits carcinogenesis in experimental animals (National Research Council, 1996). The mechanism of carcinogenesis modulation by CLA is not completely understood, although it may be related to its antioxidative properties or to the induction of apoptotic cell death and cell cycle regulation (Yamasaki *et al.*, 2006). Specific physiological effects have been linked to individual CLA isomers. The *t*10,*c*12 CLA isomer may play an important role in lipid metabolism, while the *c*9,*t*11 and the *t*10,*c*12 isomers seem to be equally effective in anticarcinogenesis (Pariza *et al.*, 2001). Since individual CLA isomers have different biological activities, the determination of the CLA isomeric profile in ruminant-derived fat is required. However, recent supplementation studies in human subjects with the *t*10,*c*12 CLA isomer revealed some adverse effects. Risérus *et al.* (2002) reported that diet supplementation with the *t*10,*c*12 CLA isomer increases oxidative stress and inflammatory biomarkers in obese men. In addition, the results obtained by Poirier *et al.* (2006) showed that the *t*10,*c*12 CLA isomer can induce inflammation of white adipose tissue. Thus, as a natural dietary

component, CLA isomers require special attention regarding the quantity consumed, and supplementation values remain a controversial issue.

Large differences in the values estimated for dietary total CLA intake among several populations have been reported (Collomb *et al.*, 2006). Although a range of strategies has been used to estimate total CLA intake, the rigorous assessment of CLA consumption requires documentation of its content and composition in the food supply (Ma *et al.*, 1999). Products from ruminant animals, including milk, dairy products and meat, are the most important sources of CLA in the human diet (Jahreis & Kraft, 2002). It is also well established that CLA isomers are also found in non-ruminant-derived meat, fish and plants, but at a much lower content (Fritsche & Steinhart, 1998). In addition, crisps, chocolates, cakes and pastries have only negligible CLA values (Jahreis & Kraft, 2002). However, detailed information on CLA isomeric distribution in commonly consumed foods, which can only be achieved by silver ion-HPLC (Delmonte *et al.*, 2006), is limited. Thus, from the above discussion it is clear that reliable information on CLA isomer consumption in human diets is highly required. Therefore, the goal of the present study was to assess the contents of total and individual CLA isomers in the most consumed ruminant-derived foods (milk, butter, yoghurt, cheese, beef and lamb meat) by the Portuguese population. In addition, based on the knowledge of Portuguese consumption habits, the contribution of these ruminant-derived foods to the daily intake of CLA isomers was also estimated.

2.2. EXPERIMENTAL PROCEDURES

2.2.1. Reagents

Merck Biosciences (Darmstadt, Germany) supplied analytical grade and liquid chromatographic-grade chemicals. Commercial standards of specific CLA isomers (*c*9,*t*11, *t*10,*c*12, *c*9,*c*11 and *t*9,*t*11) as methyl esters were obtained from Matreya Inc. (Pleasant Gap, PA, USA). Additional standards of CLA isomers mixtures of *c*,*t*, *t*,*c* and *t*,*t*, from positions 7,9 to 12,14, were synthesised as methyl esters, using the procedure described by Destaillats and Angers (2003).

2.2.2. Sample collection and treatment

Different lots of the three most important commercial Portuguese brands (ACNielsen, 2003) of half-fat milk (*n*=30), butter (*n*=30), yoghurt (*n*=45) and cheese (*n*=45) were obtained in a regular supermarket. Half-fat milk and Flamengo cheese were selected for the present study since they represent, respectively, 70 and 50% of the correspondent product consumed by the Portuguese population (ACNielsen, 2003). Moreover, meats originated on different production systems were analysed. Beef samples were collected from young bulls produced in a typical intensive production system (*n*=14) and in a traditional (semi-extensive) production system according to Protected Designation of Origin (PDO) specifications (*n*=27). Finally, lamb meat samples (*n*=8) were collected from animals reared in a typical extensive production system. All meat samples were removed from the ribeye portion (T1–T3) of animals' *longissimus dorsi*, 2–3 day after slaughter (+1 ºC), ground using a food processor (3×5 s), vacuum packed and stored at -70 ºC until required. Meat and yoghurt samples were lyophilised (–60 ºC and 2.0 hPa) to constant weight using a lyophilisator (Edwards Modulyo; Edwards High Vacuum International, Crawley, West Sussex, UK), maintained exsiccated at room temperature and analysed within 2 weeks.

2.2.3. Lipid extraction and esterification

Lipid extraction from fresh (milk, butter and cheese) and lyophilised (meat and yoghurt) samples was performed using the procedures described by Fritsche *et al.* (2000), except for milk whose extraction was based on the protocol described by Mir *et al.* (1999). Briefly, fat was extracted three times with methylene chloride–methanol (4:1, v/v) and a fourth time with n-hexane (Fritsche *et al.*, 2000). For milk, a volume of 6 ml was extracted with isopropanol (1×) followed by n-hexane (3×). Methyl esters of CLA isomers were obtained by base-catalysed transesterification (Park *et al.*, 2001b) with sodium methoxide for 2 h at 30 ºC. Total lipids were measured gravimetrically, in duplicate, by weighting the fatty residue obtained after solvent evaporation.

2.2.4. Determination of individual conjugated linoleic acid isomers

The methyl esters of CLA isomers were individually separated by triple silver-ion columns in series (ChromSpher 5 Lipids, 250 mm × 4.6 mm i.d., 5 mm particle size; Chrompack, Bridgewater, NJ, USA), using an HPLC system (Agilent 1100 Series; Agilent Technologies

Inc., Palo Alto, CA, USA) equipped with an autosampler and a diode array detector adjusted to 233 nm, according to the procedure reported previously (Alfaia *et al.*, 2006). The identification of the individual CLA isomers was achieved by comparison of their retention times with commercial and prepared standards, as well as with values published in the literature (Fritsche *et al.*, 2001). In addition, the identity of each isomer was controlled by the typical UV spectra of CLA isomers from the diode array detector in the range from 190 to 360 nm, using the spectral analysis of Agilent Chemstation for LC 3D Systems rev. A.09.01 (Agilent Technologies, 2001). Total and individual CLA isomer contents in foods were determined based on the external standard technique (using *c*9,*t*11, *t*10,*c*12, *c*9,*c*11 and *t*9,*t*11 as representatives of each geometric group of CLA isomers) and on the method of area normalisation (AOAC 963.22, 2000). The CLA isomers were expressed in gravimetric contents (mg/g product and mg/g fat) or as a percentage of the total CLA isomers identified (% of CLA isomers).

2.2.5. Estimation of the daily intake of conjugated linoleic acid isomers

The estimation of the daily intake of total and individual CLA isomers was calculated by multiplying the CLA contents (determined as described earlier) with the consumption values (*per* individual and *per* day) of each product. The consumption values were obtained from national statistics, regarding the year 2003 (INE, 2003).

2.3. RESULTS AND DISCUSSION

2.3.1. Contents of conjugated linoleic acid isomers in commercial ruminant-derived foods

Data on the total (mg/g product) and specific (mg/g fat) CLA contents and its individual isomers (% of CLA isomers) in the most consumed Portuguese ruminant-derived foods are displayed in Table 2.1.

Table 2.1 Values of total (mg/g product) and specific (mg/g fat) CLA contents, and its isomeric distribution (% of CLA isomers), in the most consumed ruminant-derived milk, dairy products and meats by the Portuguese population.

		Milk and dairy products								Meats					
		Milk (*n*=30)		Butter (*n*=45)		Yogurt (*n*=45)		Cheese (*n*=45)		Intensively produced beef (*n*=14)		Traditionally produced beef (*n*=27)		Lamb meat (*n*=8)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	Total content (mg/g product)	0.11[†]	0.045	2.64	0.789	0.06	0.022	1.09	0.313	0.06	0.050	0.08	0.052	0.95	0.196
	Specific content (mg/g fat)	7.22	2.982	4.97	0.448	4.00	1.031	4.86	1.546	4.45	1.908	4.99	1.827	11.29	3.065
% CLA isomers[††]	*t*12,*t*14	0.92	0.193	0.99	0.492	0.82	0.228	1.02	0.457	0.55	0.422	0.48	0.346	1.10	0.200
	*t*11,*t*13	1.00	0.395	1.99	0.981	1.87	0.341	2.43	0.890	0.57	0.352	1.03	0.258	1.80	0.512
	*t*10,*t*12	0.52	0.455	1.45	0.310	1.69	0.452	1.20	0.635	1.04	0.480	0.59	0.651	0.74	0.121
	*t*9,*t*11	2.21	3.159	1.58	0.267	2.04	0.244	1.49	0.330	1.16	0.598	2.14	0.926	2.17	0.082
	*t*8,*t*10	1.29	1.225	0.91	0.244	1.02	0.237	0.74	0.364	0.37	0.501	0.38	0.268	0.37	0.045
	*t*7,*t*9	1.46	2.579	0.87	0.152	1.10	0.098	0.79	0.248	15.03	14.075	0.81	0.725	0.56	0.091
	*t*6,*t*8	0.46	0.352	0.35	0.100	0.47	0.109	0.35	0.210	n.d.	n.d.	0.23	0.411	0.04	0.050
	Total *trans,trans*	7.85	3.932	8.13	1.116	9.01	0.709	8.02	0.532	18.71	13.122	5.65	1.434	6.77	0.605
	c/*t*12,14	0.18	0.105	0.31	0.326	n.d.	n.d.	n.d.	n.d.	1.21	1.232	1.35	1.615	0.64	0.157
	*t*11,*c*13	1.21	0.309	0.68	0.546	1.06	0.893	2.13	0.801	1.26	1.765	1.22	1.228	6.86	2.543
	*c*11,*t*13	0.23	0.120	0.16	0.251	n.d.	n.d.	0.10	0.169	1.10	1.761	0.72	0.974	0.15	0.129
	*t*10,*c*12	0.64	0.205	0.64	0.624	n.d.	n.d.	0.01	0.099	3.79	2.017	2.12	1.703	0.22	0.333
	*c*9,*t*11	79.21	8.442	77.77	2.857	69.74	4.822	73.70	5.288	59.89	13.683	78.35	6.315	77.31	2.027
	*t*7,*c*9*	10.21	8.917	12.32	3.188	20.20	5.359	16.03	5.846	12.09	5.206	9.17	3.885	8.04	4.364
	Total *cis/trans*	91.67	3.950	91.87	1.116	90.99	0.709	91.98	0.532	80.21	13.818	92.93	1.974	93.23	0.605
	*c*11,*c*13	0.48	0.206	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	*c*10,*c*12	<0.01	<0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	*c*9,*c*11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.08	1.273	1.42	1.226	n.d.	n.d.
	*c*8,*c*10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Total *cis,cis*	0.48	0.207	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.08	1.273	1.42	1.226	n.d.	n.d.

*This CLA isomer co-eluted with minor amounts of the *t*8,*c*10 isomer; [†]Values expressed in mg/ml product; [††]Values of each isomer as percentage of the total CLA isomers identified in each product; n.d. = not detected.

The highest total CLA concentration was found in butter (2.64 mg/g product) and cheese (1.09 mg/g product) due to the high fat content present in these products. Lamb meat was the third richest product in total CLA (0.95 mg CLA/g meat). Yoghurt and intensively produced beef depicted residual CLA contents (0.06 mg/g product). CLA specific contents among meats ranged from 4.45 (intensively produced beef) to 11.29 mg/g fat (lamb meat). While milk showed a still remarkably high specific CLA content (7.22 mg CLA/g fat), the remaining products displayed considerably lower but similar concentrations, ranging from 4.00 to 4.97 mg/g fat. Lamb meat is usually originated from grass feeding systems and it is well known that the inclusion of grass in the diet improves CLA content in meat (French *et al.*, 2000; Alfaia *et al.*, 2007). Compared with available data, Chin *et al.* (1992) encountered a concentration of 5.5 mg CLA/g fat in homogenised milk. The specific CLA contents in butter and yoghurt were similar to those reported by Ma *et al.* (1999) (4.7 and 4.4 mg/g fat, for butter and yoghurt, respectively). For yoghurt, Chin *et al.* (1992) reported 4.8 mg CLA/g fat, whereas Lin *et al.* (1995) found a concentration of 3.8 mg CLA/g fat. Regarding cheese, Shantha *et al.* (1992) measured CLA contents varying from 3.2 to 8.9 mg/g fat, an interval that includes our calculated concentration of 4.86 mg CLA/g fat. Chin *et al.* (1992) reported concentrations ranging from 2.7 mg CLA/g fat in veal to 4.3 mg CLA/g fat in fresh ground beef, this interval being close to the concentrations found in the present study for intensive and traditional meat samples. Also, Shantha *et al.* (1992) described variations in CLA contents in raw steaks (ribeye, round, t-bone and sirloin) varying from 3.1 to 8.5 mg/g fat. The differences reported on CLA content in ruminant fatty acid composition may be explained by the influence of dietary factors (production system) and, to a lesser extent, by genetic factors (De Smet *et al.*, 2004). French *et al.* (2000) reported that meat fat from grazing steers displays higher CLA contents (10.8 mg/g fatty acids) than those obtained from animals fed concentrate (3.7 mg/g fatty acids). Additionally, discrepancies in the CLA content between different animal tissues, different breeds or upbringing, and even within the same breed, have already been reported and reviewed by Schmid *et al.* (2006). Regarding CLA in dairy products, Collomb *et al.* (2002, 2004) concluded that the quality of milk and ripened cheese is influenced by many factors, including the composition of fodder consumed and the altitude at which the cow grazes. The CLA isomeric profile of the analysed foods is presented in Table 2.1. In general, CLA distribution showed a clear predominance of the bioactive *c*9,*t*11 isomer (59.89–79.21%), followed by the *t*7,*c*9 isomer (8.04–20.20%), which co-eluted with minor amounts of the *t*8,*c*10 isomer. Similarly to total CLA content, diet is the major factor that affects the profile of CLA in ruminant fats (Dannenberger *et al.*, 2005; De La Torre *et al.*, 2006). Moreover, many of the differences in CLA profile appear to be related to pasture

versus concentrate feeding. In the present study, lamb meat (pasture fed) showed higher percentages of the *t*11,*c*13, *t*11,*t*13 and *t*12,*t*14 isomers and lower of the *t*7,*c*9 isomer, when compared with intensively and traditionally produced beefs. These differences may be explained by distinct grass intake of the animals since it was shown that pasture feeding, compared with concentrate feeding, increases the proportion of the *t*11,*c*13, *t*11,*t*13 and *t*12,*t*14 isomers and decreases the percentage of the *t*7,*c*9 isomer in beef lipids (Dannenberger *et al.*, 2005). Based on these results, Dannenberger *et al.* (2005) suggested that the *t*11,*c*13, *t*12,*t*14 and *t*11,*t*13 CLA isomers are sensitive grass intake indicators. Breed and muscle type have also been reported as determinant of *t*11,*c*13 isomer percentage in beef lipids (Dannenberger *et al.*, 2005). Regarding the CLA profile of intensively produced beef, the most abundant isomer was the *c*9,*t*11 (59.89%) followed, in decreasing order, by the *t*7,*t*9 isomer (15.03%) and the *t*7,*c*9 isomer (12.09%). The *t*7,*c*9 isomer is mentioned frequently as the second most prevalent CLA isomer (Yurawecz *et al.*, 1998) and, like the most abundant *c*9,*t*11 isomer, its content in milk and tissues mainly results from endogenous synthesis through the Δ9-desaturation of the rumen-derived *trans*-octadecenoate precursor (Palmquist *et al.*, 2004). With the exception of the *c*9,*t*11 and *t*7,*c*9 isomers, the origin of all other CLA isomers is the ruminal biohydrogenation of dietary unsaturated C18 fatty acids, although the metabolic pathways producing these compounds are not yet elucidated (Collomb *et al.*, 2004). The *t*10,*c*12 CLA isomer, which apparently affects lipid metabolism (Pariza *et al.*, 2001), was present in very small proportions (0.01–2.12%), reaching the highest levels in beef, from either intensive or semi-extensive production systems. In the *trans*,*trans* region, the most abundant isomer was the *t*9,*t*11, ranging from 1.16 to 2.21% of total CLA. The sums of *trans*,*trans* (5.65–8.01%) and *cis*/*trans* (90.99–93.23%) CLA isomers were similar in all analysed products, except for the intensively produced beef, which were 18.71 and 80.21% for *trans*,*trans* and *cis*/*trans*, respectively. The sum of the *cis*,*cis* CLA isomers only showed residual contents for all analysed products (<1.42%).

2.3.2. Estimation of the daily intake of conjugated linoleic acid isomers

Table 2.2 reports an estimation of the contribution of ruminant derived foods for the daily intake of Portuguese consumers, *per* individual, of total and individual CLA isomers. Usually dietary intake is assessed by 3 or 7 days dietary records, representing the diet of a medium-term period, or by food-frequency questionnaires, expected to reflect the regular diet (Ritzenthaler *et al.*, 2001).

Table 2.2 Estimation of the contribution of ruminant-derived foods to the average daily intake of total and individual CLA isomers in Portugal.

		Milk and dairy products				Meats			
		Milk	Butter	Yogurt	Cheese	Intensively produced beef	Traditionally produced beef	Lamb meat	
	Daily consumption (g)	166.17[†]	4.38	54.25	27.12	47.15	1.07	8.77	Total
CLA daily intake	(mg/day)	18.01	11.57	3.23	29.61	3.04	0.09	8.16	73.70
	(%)	24.38	15.66	4.37	40.10	4.12	0.12	11.07	100.00
CLA isomers/Total CLA intake (%)	*t*12,*t*14	0.22	0.13	0.04	0.45	0.02	<0.01	0.12	0.99
	*t*11,*t*13	0.24	0.27	0.09	1.06	0.03	<0.01	0.20	1.89
	*t*10,*t*12	0.13	0.24	0.07	0.44	0.05	<0.01	0.08	1.01
	*t*9,*t*11	0.53	0.25	0.09	0.57	0.06	<0.01	0.24	1.73
	*t*8,*t*10	0.30	0.15	0.04	0.26	0.01	<0.01	0.04	0.82
	*t*7,*t*9	0.37	0.14	0.05	0.29	0.41	<0.01	0.06	1.32
	*t*6,*t*8	0.11	0.06	0.02	0.12	0.00[††]	<0.01	<0.01	0.32
	Total *trans,trans*	1.90	1.23	0.39	3.20	0.58	0.01	0.75	8.07
	c/*t*12,14	0.05	0.06	0.00[††]	0.00[††]	0.04	<0.01	0.07	0.23
	*t*11,*c*13	0.29	0.13	0.06	0.89	0.06	<0.01	0.79	2.22
	*c*11,*t*13	0.06	0.03	0.00[††]	0.04	0.03	<0.01	0.02	0.18
	*t*10,*c*12	0.16	0.12	0.00[††]	0.01	0.13	<0.01	0.02	0.45
	*c*9,*t*11	19.34	12.29	3.09	29.83	2.73	0.10	8.59	76.10
	*t*7,*c*9*	2.46	1.81	0.82	6.13	0.50	0.01	0.59	12.56
	Total *cis/trans*	22.36	14.43	3.97	36.90	3.50	0.11	10.32	91.77
	*c*11,*c*13	0.12	0.00[††]	0.00[††]	0.00[††]	0.00[††]	0.00[††]	0.00[††]	0.12
	*c*10,*c*12	<0.01	0.00[††]	0.00[††]	0.00[††]	0.00[††]	0.00[††]	0.00[††]	0.00[††]
	*c*9,*c*11	0.00[††]	0.00[††]	0.00[††]	0.00[††]	0.04	<0.01	0.00[††]	0.04
	*c*8,*c*10	0.00[††]	0.00[††]	0.00[††]	0.00[††]	0.00[††]	0.00[††]	0.00[††]	0.00[††]
	Total *cis,cis*	0.12	0.00[††]	0.00[††]	0.00[††]	0.04	<0.01	0.00[††]	0.16

*This CLA isomer co-eluted with minor amounts of the *t*8,*c*10 isomer; [†]Values expressed in ml/day product; [††]Missing calculation due to undetected isomer in CLA profile.

Additional to these methodologies, Ritzenthaler *et al.* (2001) also conducted chemical analysis of food duplicates and concluded that daily CLA intake is underestimated by written dietary methods. In the present study, neither of these methodologies was applied. The calculation was done, as described earlier, by attending to the CLA contents determined in ruminant-derived products commercially available in Portugal (see Table 2.1) and the consumption data (*per* individual and *per* day) of each product, according to the most recent national statistics (INE, 2003). The average daily intake of total CLA was estimated as being 73.70 mg/individual. This value may be slightly underestimated since non-ruminant derived products (for example, meat, fish, plant crisps, chocolates, cakes and pastries) may have, as stated earlier, minor CLA contents (Fritsche & Steinhart, 1998; Jahreis & Kraft, 2002). The major food sources contributing to this value were cheese and milk (40.10 and 24.38%, respectively), followed, in decreasing order, by butter (15.66%) and lamb meat (11.07%). Traditionally produced beef contributed only 0.12% to the total CLA intake. Reflecting the food isomeric distribution, *c*9,*t*11 (76.10%) and *t*7,*c*9 (12.56%) were the main isomers present in the diet, as illustrated in Figure 2.1. The third most important isomer was *t*11,*c*13, contributing 2.22% to the daily intake. The *t*11,*t*13, *t*9,*t*11 and *t*7,*t*9 CLA isomers were the most relevant in the *trans*,*trans* isomers region.

Depending on the country, the estimation of average CLA or *c*9,*t*11 consumption ranges between 15 and 1000 mg (Table 2.3). Even if the major food sources of CLA remain constant, their relative contributions to dietary intake may vary with food availability and eating preferences (Schmid *et al.*, 2006). Based on milk consumption data, Wolff and Precht (2002) estimated the *c*9,*t*11 ingestion in fifteen European countries, obtaining higher intake values in North Europe and lower in Mediterranean countries. According to these authors, France and Italy showed consumption values close to the European Union average and similar daily ingestions were observed for Spain (140 mg), Greece (150 mg) and Portugal (150 mg). The difference from our values for Portugal might be explained by the distinct estimation methods and statistics sources used. In Germany, the overall estimated *c*9,*t*11 consumption reported by Fritsche and Steinhart (1998) was relatively high (350 and 430 mg/day for women and men, respectively), by one week dietary records.

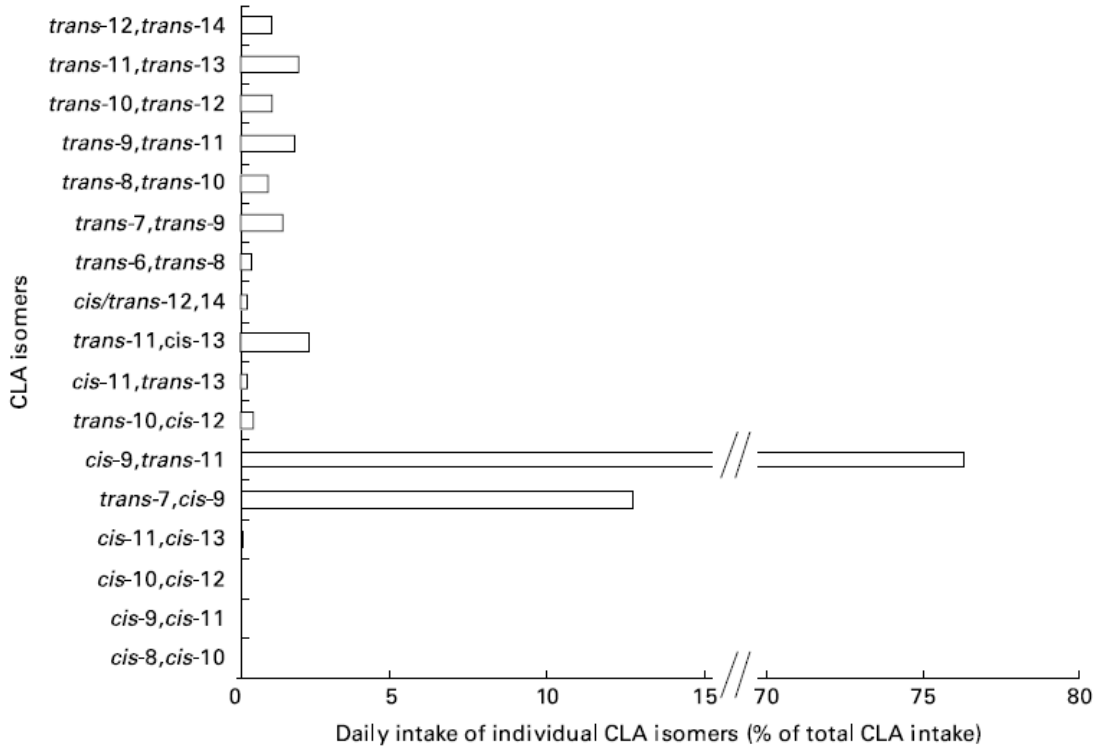

Figure 2.1 Conjugated linoleic acid (CLA) isomeric distribution of the estimated daily CLA intake for the Portuguese population.

Table 2.3 Average CLA intake (mg/day) estimated for several countries.

Country	Method*	Daily intake	Estimated isomer	Reference
European Union	Milk intake	250	*c*9,*t*11	Wolff &Precht (2002)
Portugal	Milk intake	150	*c*9,*t*11	Wolff & Precht (2002)
Spain	Milk intake	140	*c*9,*t*11	Wolff & Precht (2002)
France	Milk intake	300	*c*9,*t*11	Wolff & Precht (2002)
Italy	Milk intake	220	*c*9,*t*11	Wolff & Precht (2002)
Greece	Milk intake	150	*c*9,*t*11	Wolff & Precht (2002)
Germany	7d DR	350 - 430	*c*9,*t*11	Fritsche & Steinhart (1998)
Sweden	1d DR	160	*c*9,*t*11	Jiang *et al.* (1999)
Canada	7d DR	15 - 174	*c*9,*t*11	Ens *et al.* (2001)
USA	FFQ	93 - 197	Total CLA	Ritzenthaler *et al.* (2001)
USA	FFQ	72 - 151	*c*9,*t*11	Ritzenthaler *et al.* (2001)
USA	3d FD	151 - 212	Total CLA	Ritzenthaler *et al.* (2001)
USA	3d FD	140 - 193	*c*9,*t*11	Ritzenthaler *et al.* (2001)
USA	3d DR	104 - 176	Total CLA	Ritzenthaler *et al.* (2001)
USA	3d DR	79 - 133	*c*9,*t*11	Ritzenthaler *et al.* (2001)
USA	3d DR	127	*c*9,*t*11	Herbel *et al.* (1998)
Australia	not reported	500-1000	Total CLA	Parodi (1994)

* The methods for CLA intake estimation were based on either the estimation of milk intake (milk intake), dietary records (DR), food duplicates (FD) or food-frequency questionnaires (FFQ).

Based on the same method, an assessment of *c*9,*t*11 intake in a small group of young Canadians determined an average ingestion of 94.9 mg/day, ranging between 15 and 174 mg/day (Parodi, 1994), which is not very far from our determination for this particular isomer

(see Table 2.2). Ritzenthaler *et al.* (2001) reported that young men and women living in the USA consumed about 151–212 and 140–193 mg/day for total CLA and *c*9,*t*11, respectively, by 3 days food duplicates. In agreement with that study, Herbel *et al.* (1998) reported, for the same country and by using the same methodology, the daily intake of 127 mg CLA. McGuire *et al.* (1999) advanced two possible reasons for the higher CLA intake for Germany compared with those for USA: more fat consumption in Germany and vast food nutrient database used. In Table 2.3, Australia shows the highest CLA consumption, 500–1000 mg/day (Parodi, 1994), although the estimation method applied is unknown. Nutritional habits are also dependent on sex, although the lack of consumption statistics *per* woman and man did not allow taking this variable into account in the present study. Additionally, the present study differs from its counterparts presented in Table 2.3 because CLA concentration in food was determined by Ag^{+}-HPLC, in contrast to GC.

One of the issues related to CLA importance in human nutrition is related to its anticarcinogenic activity (Yamasaki *et al.*, 2006). Levels of CLA as low as 0.1% in the diet have been seen as sufficient to produce a significant decrease in mammary tumour yield in rats challenged with a low dose of 7,12-dimethylbenz[a]anthracene (Ip *et al.*, 1994). The daily CLA intake in Portugal is 0.0038% (intake of 1916.9 g food/day based on Portuguese statistics from Instituto Nacional de Estatística (1997)), which only represents 3.8% of the above recommended value. Moreover, at a 0.1% dosage of CLA, a 300 g rat will consume approximately 0.015 g CLA/day. Extrapolating directly to a 70 kg man or woman, CLA consumption *per* day has to equal 3 g to confer a similar health benefit (Ip *et al.*, 1994). However, differences in metabolic rate (particularly, in lipid metabolism) between these two species require a more suitable extrapolation from a rat model to man, using the metabolic weight (Terpstra, 2001). Such extrapolation indicates that 0.8 g CLA/day would be protective in man. Therefore, on the basis of the anticancer effects of CLA in rats, as experimental models, a daily consumption of 0.8–3.0 g CLA might provide a significant human health benefit (Parish *et al.*, 2003). One of the few epidemiological studies relating the incidence of breast cancer in postmenopausal Netherlands women and levels of CLA intake showed a positive, although weak, correlation (*P* for trend=0.02) between 200 mg CLA intake *per* day and this cancer occurrence (Voorrips *et al.*, 2002). Another study associated high fat consumption, including CLA, with colorectal cancer incidence in Swedish women (*P* for trend=0.002) aged 40–76 years (Larsson *et al.*, 2005). These authors concluded that high intakes of high fat dairy foods, containing at least 127.8 mg CLA/day, may reduce the risk of colorectal cancer. Actually, in several countries, levels as high as the above-mentioned are

consumed (see Table 2.3). However, it is well known that cancer is a multifactorial disease and, therefore, many other factors besides diet components still determine its occurrence. As being so, facing the relatively low ingestion value for Portugal presented in the present study (73.70 mg CLA/day), no preventive carcinogenic effects of CLA are expected to be found for the Portuguese population. A possible solution in order to reach beneficial values of CLA in the diet is through supplementation. Of note, dietary supplements have a different CLA isomeric profile compared with foodstuffs. The main difference concerns the high percentage of *t*10,*c*12 in supplements and some studies have recently demonstrated adverse effects of this isomer on human health (Risérus *et al.*, 2002). Several authors have used indirect methodologies to estimate both typical and extreme intakes of CLA in a limited number of populations. However, in the present study we present an unparalleled and detailed overview of CLA isomeric profile in dairy and meat products, which allowed the direct estimation of daily CLA intake for the Portuguese population. In human trials, synthetic CLA supplements are usually used and these do not reflect the natural isomeric composition in foodstuffs. Whether natural CLA sources (meat, milk and its derivatives from ruminant animals) have a similar impact on human health warrants further research (for a review, see Schmid *et al.*, 2006). Essentially, examination of the relationships among dietary intake of CLA isomers, their contents in adipose tissue and plasma and risk of various chronic degenerative diseases (for example, cancer, diabetes, and atherosclerosis) is essential for scientists and public health officials to draw conclusions concerning the importance of dietary CLA (and its isomers) to human health. Studies are actually in progress to clarify these questions. Therefore, enhancing our knowledge concerning CLA isomeric profile in various populations must remain a primary focus for research in this area.

2.4. CONCLUSIONS

In the present study, contents of total and individual CLA isomers in various Portuguese ruminant-derived foods were assessed. Regarding milk and dairy products, total CLA contents ranged from 4.00 mg/g fat in yoghurt to 7.22 mg/g fat in butter, while in meats these concentrations varied from 4.45 mg/g fat in intensively produced beef to 11.29 mg/g fat in lamb meat. The most abundant CLA isomers in these products were *c*9,*t*11 (59.89-79.21%) and *t*7,*c*9 (8.04-20.20%). The average total CLA intake for the Portuguese population was calculated to be 73.70 mg/day. Moreover, milk and cheese are believed to be the two products that contributed the most to the final CLA intake. Finally, as a result from the food isomeric

distribution, the *c*9,*t*11 and *t*7,*c*9 are the most represented CLA isomers, with, respectively, 76.10 and 12.56% of the total intake value.

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CHAPTER 3 Diet supplementation with the *cis*9,*trans*11 conjugated linoleic acid isomer affects the size of adipocytes in Wistar rats

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Abstract

Previous reports have demonstrated that conjugated linoleic acid (CLA) acts on body fat accumulation in a variety of animal models. The aim of the present study was to investigate the effect of *c*9,*t*11 and *t*10,*c*12 CLA isomers on the number and size of adipocytes from the epididymal and retroperitoneal fats in Wistar male rats. A 5.1% palm oil based diet was supplemented with CLA isomers as follows: 0.6% of *c*9,*t*11, 0.6% of *t*10,*c*12, 1.3% of *c*9,*t*11 and *t*10,*c*12 isomers in mixture, and a control non-supplemented group for comparative purposes. Fat depots were prepared on microscope slides for histologic examination using an image-analysis software to count the number of adipocytes and measure cell sizes. The results showed that CLA isomers did not affect (P>0.05) either final body and fat depot weights or serum lipids (with the exception of triacylglycerols) and adipokines (leptin and adiponectin). Animals fed the *c*9,*t*11 CLA isomer diet showed larger adipocytes when compared to other groups. Independently of the CLA dietary treatment, retroperitoneal fat showed larger adipocytes (3319 μm^2) and therefore a smaller number of adipocytes *per* unit of area, compared to epididymal fat (3055 μm^2). Taken together, the data suggest that a palm oil based diet supplemented with the *c*9,*t*11 CLA isomer in Wistar rats, in contrast to the *t*10,*c*12 isomer and the mixture of both isomers, increases adipocyte dimensions in epididymal and retroperitoneal fat depots, while having a minor effect in serum lipids and adipokines.

[a] These authors contributed equally

3.1. INTRODUCTION

Conjugated linoleic acid (CLA) is a group of naturally occurring fatty acids produced by fermentative bacteria in the rumen of polygastric animals, due to linoleic acid isomerisation. Another source of CLA is the endogenous Δ9-desaturation of vaccenic acid (Griinari and Bauman, 1999). Twenty four different CLA isomers have been identified as occurring naturally in ruminant fat (Cruz-Hernandez *et al*., 2004). The CLA isomeric profile of meat and dairy products presents at least 60% of the *c*9,*t*11 isomer (Martins *et al.*, 2007), whereas the content in the *t*10,*c*12 isomer is only vestigial. In contrast, the commercial synthetic CLA mixtures contain equal amounts of *c*9,*t*11 and *t*10,*c*12 isomers (Larsen *et al.*, 2003). These two CLA isomers have been associated with beneficial health properties, such as anticancerogenesis (Ip *et al.*, 1991) and anti-atherogenesis (Kritchevsky *et al.*, 2003). In addition, numerous reports have demonstrated that dietary CLA can interfere with body composition, reducing the accumulation of adipose tissue in several mammalian species (Domeneghini *et al.*, 2006).

The distribution of white adipose tissue in mammals depends on both genetic and environmental factors. Specifically, it depends on the number of adipocytes, as well as on the metabolic status of the animal and the degree of filling with depot fat (Domeneghini *et al.*, 2006). The main function of adipocytes is to store triacylglycerols during periods of energy excess and to mobilise this reserve when expenditure exceeds intake. In addition to their energy-storage function, adipocytes are connected to the vascular network and have an important endocrine role. Adipocytes secrete several adipokines, such as leptin and adiponectin, therefore modulating energy and lipid metabolisms (Brown & McIntosh, 2003). Adiponectin and leptin are believed to display antagonistic physiologic effects in animals. Adiponectin is considered a defensive cytokine presenting important protective effects, such as anti-atherogenic, antidiabetic, and anti-inflammatory properties (Matsuzawa, 2006). Leptin is an controls food intake and energy expenditure, being present at elevated circulating levels in obese humans (Savage & O´Rahilly, 2001). The capacity of different individual CLA isomers to influence body composition and adipokine metabolism in humans is still not clearly established. The conflicting results reported in the literature may result from distinct isomer-specific mechanisms, with the *t*10,*c*12 CLA isomer being possibly responsible for most of the anti-adipogenic effects (Evans *et al.*, 2002). This observation highlights the need to study the influence of CLA isomers separately, thereby disabling the possibility of interactions between isomers that contribute to masking individual biological effects. As CLA

isomers are commercially available for anti-adipogenic purposes, further studies on these conjugated fatty acid properties are of extreme relevance for human nutrition research. However, the influence of CLA on body fat is still far from well characterised and some contradictory effects have been reported (Brown & McIntosh, 2003; Evans *et al.*, 2002). In addition, although some studies have reported that CLA affects the histologic properties of fat tissue (Azain *et al.*, 2000; Poulos *et al.*, 2001; Corino *et al.*, 2005; Simón *et al.*, 2005; Noto *et al.*, 2007), there is no information regarding the interaction between individual isomers in rats. Thus, this article aims to describe and compare the histologic effects of different dietary CLA isomers, individually and combined, through a histometrical analysis to quantify the size and number of adipocytes *per* area, both on epididymal and on retroperitoneal fat depots. Diets were formulated as saturated based on mimic patterns of human nutrition from the modern societies. Moreover, CLA seems to be more beneficial in controlling adiposity when supplemented in a diet rich in saturated *vs*. unsaturated fats (Kloss *et al.*, 2005). Therefore, the experimental model selected for this study was the Wistar rat fed on a 5.1% palm oil based diet supplemented with 1.3% of a CLA mixture (equal proportions of *c*9,*t*11 and *t*10,*c*12 isomers) and 0.6% of the individual *c*9,*t*11 and *t*10,*c*12 CLA isomers. To clarify the possible mechanisms involved in CLA metabolism of adipocytes, the serum concentrations of leptin and adiponectin were also determined. In summary, it was hypothesised that saturated based diets supplemented with *c*9,*t*11 and *t*10,*c*12 CLA isomers would contribute to altering adipose mass and fat cell size and, therefore, change the level of circulating adipokines.

3.2. EXPERIMENTAL PROCEDURES

3.2.1. Diet oils

The CLA mixture oil with 80% purity and identical proportions of *c*9,*t*11 and *t*10,*c*12 isomers was a generous gift from PharmaNutrients, Inc (Gurnee, Ill). The *c*9,*t*11 and *t*10,*c*12 CLA oils with 90% purity were supplied by Natural ASA (Hovdebygda, Norway). Palm and sunflower oils were purchased from Sigma-Aldrich, Ltd (St Louis, Mo).

3.2.2. Wistar rats and diets

The experimental protocol was reviewed by the ethics committee of CIISA/FMV and approved by the Animal Care Committee of the National Veterinary Authority (Direcção Geral de Veterinária, Portugal) following the appropriate European Union guidelines (N.

86/609/EEC). Thirty nine male Wistar rats (Harlan Interfauna Iberica, SL, Barcelona, Spain) aged 28 days were housed individually in an environmentally controlled room with temperature fixed at 22 °C and a photoperiod of 12 h (lights out at 7:00 pm). After arrival, rats were acclimatised and fed a commercial standard diet (Harlan Teklad Global Diets 2014, Oxon, England) without CLA for one week. After this period, rats were assigned to four body weight–matched groups, of ten animals each, with the exception of the CLA mixture group, which had nine animals. For the experiments, the standard pellets (Harlan Teklad Global Diets 2014, Oxon, England) were enriched with palm, sunflower, and CLA oils (w/w), as follows: control group - 5.1% of palm oil plus 1.3% of sunflower oil; mixture group - 5.1% of palm oil plus 1.3% of CLA mixture oil; *c*9,*t*11 group - 5.1% of palm oil plus 0.6% of *c*9,*t*11 CLA oil; *t*10,*c*12 group - 5.1% of palm oil plus 0.6% of *t*10,*c*12 CLA oil. Considering the CLA oil purity, the final concentration of CLA isomers in the diets was 1% for the mixture group and 0.5% for both the *c*9,*t*11 and the *t*10,*c*12 groups. Table 3.1 presents the composition of the experimental diets. The diets were provided *ad libitum* during 8 weeks. Body weight and feed intake were measured twice a week. At the end of the experimental period, rats were fasted for 12 h and killed by decapitation, under light isofluorane (Abbott, Abbott Park, Ill) anesthesia. The trunk blood was centrifuged (3000 rpm for 10 min at room temperature) to separate the serum. The serum was analysed within 24 h at a clinical chemistry laboratory—Clínica Médica e Diagnóstico Dr. Joaquim Chaves (Algés, Portugal). After blood collection, epididymal and retroperitoneal fat depots were excised and weighted. For histometrical analyses, samples from each fat depot (approximately 100 mg) were fixed by immersion in 10% neutral buffered formalin (Merck, Darmstadt, Germany) for 24 h and processed for paraffin (Microscopy Histosec, Merck) embedding.

3.2.3. Fatty acid composition of the diets

Diet fatty acids were directly converted to methyl esters (fatty acid methyl esters, FAME) by a combined procedure of methylation, base catalysis followed by acid catalysis, according to the method of Christie *et al.* (2001), slightly modified by Raes *et al.* (2004). Briefly, 1 ml of dry toluene was added to 0.25 g of lyophilised diet and fatty acids were methylated with sodium methoxide in anhydrous methanol (0.5 mol/l) for 30 minutes, followed by hydrogen chloride in methanol (1/1 v/v) for 10 minutes, at 50 °C. Fatty acid methyl esters were extracted twice with 3 ml of hexane and pooled extracts were evaporated, until 2 ml, at 35 °C under nitrogen. Gas chromatography analysis of fatty acid methyl esters was performed using a fused-silica capillary column (CP-Sil 88; 100 m × 0.25 mm id; Chrompack, Varian, Inc,

Palo Alto, Calif), equipped with a flame-ionisation detector, as described by Bessa *et al.* (2007).

Table 3.1 Composition of experimental diets.

	Control	**Mixture**	***c*9,*t*11**	***t*10,*c*12**
Proximate composition				
Dry matter (%)	93.0	93.0	93.0	93.0
Crude protein (% DM)	16.2	16.2	16.2	16.2
Crude fat (% DM)	8.6	8.6	8.6	8.6
Fibre (% DM)	5.3	5.3	5.3	5.3
Ash (% DM)	5.6	5.6	5.6	5.6
Gross energy (kJ/g)	15.1	15.1	15.1	15.1
Fat ingredients				
Soybean oil (% DM)	2.3	2.3	2.3	2.3
Sunflower oil (% DM)	1.3	0.0	0.7	0.7
Palm oil (% DM)	5.1	5.1	5.1	5.1
CLA oil (% DM)	0.0	1.3	0.6	0.6
Fatty acid profile (% FAME)				
14:0	0.6	0.7	0.7	0.7
16:0	33.0	35.9	34.3	34.1
18:0	3.4	3.3	3.4	3.3
18:1	32.0	30.2	31.2	31.0
18:2*n*-6	29.6	23.3	26.7	27.0
18:3*n*-3	1.3	1.4	1.4	1.4
*c*9,*t*11 CLA	0.0	2.5	2.3	0.1
*t*10,*c*12 CLA	0.0	2.6	0.2	2.4

DM, dry matter; FAME, fatty acid methyl esters.

3.2.4. Serum lipids and adipokines

Total cholesterol (CHOD-PAP, Roche Diagnostics), HDL-cholesterol (HDL-C plus, Roche Diagnostics), LDL-cholesterol (LDL-C plus, Roche Diagnostics), and triacylglycerols (GPO-PAP, Roche Diagnostics) were analysed in serum through diagnostic test kits (Roche Diagnostics, Mannheim, Germany) using a Modular Hitachi Analytical System (Roche Diagnostics). Serum adipokines were measured using commercial ELISA kits from R&D (MOB00, Minneapolis, Minn) for leptin and from Linco Research (EZRADP-62K, St Louis, Mo) for adiponectin.

3.2.5. Histometrical examinations

Three serial tissue sections (10 μm thick) were cut on a microtome (Leica, SM 2000R, Nussloch, Germany) from each of the paraffin-embedded specimens. Sections were stained with hematoxylin (Bio-optica, Milan, Italy) and eosin (Richard-Allan Scientific, Kalamazoo, Mich) to assess morphology under a light microscope (Olympus BX51 equipped with a DP11 microscope digital camera system, Olympus, Tokyo, Japan). For histometry, the area (μm^2) of 100 adipocytes from each section was determined under the microscope (magnification ×100), using the DP software for image analysis (Olympus DP-Soft version 3.0 for Windows 95/98). The number of adipocytes was also determined in 5 fields *per* section at a magnification of ×100. The entire histologic plan was followed as described by Corino *et al.* (2005).

3.2.6. Statistics

Values are presented as mean ± SEM for data concerning body composition, biochemical, and histologic parameters. Data statistical analysis was performed using the Statistical Analysis System (SAS Institute, Cary, NC) (SAS, 2004). The body composition and biochemical serum parameters were analysed by the general linear model (procGLM) to perform a one-way analysis of variance. The analysis of variance of histometrical data was performed using the mixed model, procMIXED, where diet and type of fat depot constituted fixed effects and rat constituted a random effect. The mixed model was selected because the two fats studied were collected from the same animal. To better understand the adipocyte size distribution, histograms were constructed. The Sturges' rule (Sturges, 1926) was applied to define the number of classes. Assuming normal distributions, classes containing the mean were presented at the histogram center. Differences between least square means were determined by Tukey's test. A *P* value less than 0.05 was assumed for statistically significant differences (Zar, 1999).

Table 3.2 Effects of dietary conjugated linoleic acid and its individual isomers on growth performance, tissue weights, and serum lipids and adipokines.

	Control	Mixture	c9,t11	t10,c12
Growth performance (g)				
Initial body weight	114.5 ± 2.10	114.8 ± 1.98	114.9 ± 2.03	114.9 ± 1.86
Final body weight	355.0 ± 4.51	336.7 ± 3.75	344.6 ± 6.50	347.5 ± 4.83
Weight gain	240.6 ± 5.11	222.4 ± 3.59	229.7 ± 7.16	232.6 ± 4.75
Daily weight gain	4.5 ± 0.13	4.1 ± 0.11	4.3 ± 0.17	4.3 ± 0.11
Total intake	1205.0 ± 18.81	1136.8 ± 12.84	1203.0 ± 26.49	1204.5 ± 16.94
Daily intake	22.2 ± 0.41	20.9 ± 0.22	22.1 ± 0.59	22.1 ± 0.34
Food efficiency	0.2 ± 0.004	0.2 ± 0.004	0.2 ± 0.005	0.2 ± 0.004
Tissue weights (g)				
Liver	9.34 ± 0.216	9.28 ± 0.277	9.57 ± 0.347	9.43 ± 0.244
Spleen	0.56 ± 0.031	0.61 ± 0.024	0.58 ± 0.034	0.60 ± 0.034
Epididymal fat	6.68 ± 0.584	6.70 ± 0.391	6.46 ± 0.617	6.86 ± 0.520
Retroperitoneal fat	5.58 ± 0.816	4.94 ± 0.479	6.81 ± 0.930	5.61 ± 0.866
Serum lipids (mg/dl)				
Total cholesterol	90 ± 6.7	82 ± 4.5	84 ± 5.6	87 ± 5.0
HDL-cholesterol	71 ± 4.1	64 ± 3.3	65 ± 3.3	68 ± 3.4
LDL-cholesterol	16 ± 2.2	12 ± 1.4	12 ± 1.8	12 ± 1.5
VLDL-cholesterol †	12 ± 1.8	15 ± 1.7	21 ± 4.2	14 ± 1.3
Triacylglycerols	58^{a} ± 9.1	$76^{a,b}$ ± 8.3	107^{b} ± 20.9	$70^{a,b}$ ± 6.5
Total lipids ‡	387 ± 20.1	390 ± 12.3	426 ± 26.2	393 ± 13.4
Serum adipokines				
Leptin (pg/ml)	847 ± 110.1	809 ± 102	1064 ± 256.4	826 ± 101.8
Adiponectin (μg/ml)	22 ± 1.4	29 ± 2.8	21 ± 2.2	26 ± 2.3

Data represent mean values ± SEM. ^{a,b}Values with different superscript roman letters are significantly different according to one-way analysis of variance with Tukey's test, at $P<0.05$. † VLDL-cholesterol = 1/5 [triacylglycerols] (Friedewald *et al.*, 1972). ‡ Total lipids = [total cholesterol] × 1.12 + [triacylglycerols] × 1.33 + 148 (mg/dl) (Covaci *et al.*, 2006).

3.3. RESULTS

Table 3.2 presents the overall characterisation of the studied animal groups, in terms of growth performance, tissue weight, and serum lipid profile. There were no significant differences ($P>0.05$) among the dietary groups in relation to feed intake, body weight, and weight gain during the experimental period. In addition, dietary treatments had no effect on feed conversion efficiency and tissue weights, including epididymal and retroperitoneal fat depots. However, the *c*9,*t*11 CLA isomer treatment increased serum triacylglycerol levels, although no other variations on lipid profile were observed. Data concerning the histometrical evaluation of epididymal and retroperitoneal white fat depots, as affected by different CLA dietary treatments, are summarised in Table 3.3, and exemplified in Figure 3.1.

Table 3.3 Effects of dietary conjugated linoleic acid and its individual isomers on adipocytes area (μm^2) and number (in 360×10^3 μm^2) from epididymal and retroperitoneal fat depots.

	Diet					Fat			*P*		
	Control	**Mixture**	**c9,t11**	**t10,c12**	**SEM[1]**	**EPI**	**RP**	**SEM**	**Diet**	**Fat**	**Diet × Fat**
Area	3077 [a]	2999 [a]	3721 [b]	2951 [a]	150.8	3055	3319	93.6	0.029	0.020	0.307
Number	121 [ab]	132 [a]	108 [b]	133 [a]	5.5	131	117	3.4	0.010	0.001	0.672

Data represent mean values ± SEM. [a,b]Values with different superscript roman letters are significantly different according to one-way analysis of variance with Tukey's test, at $P<0.05$. EPI, epididymal; RP, retroperitoneal. [1]Values of SEM for the CLA mixture group ($n=9$) are 59.0 and 5.8 for area and number, respectively.

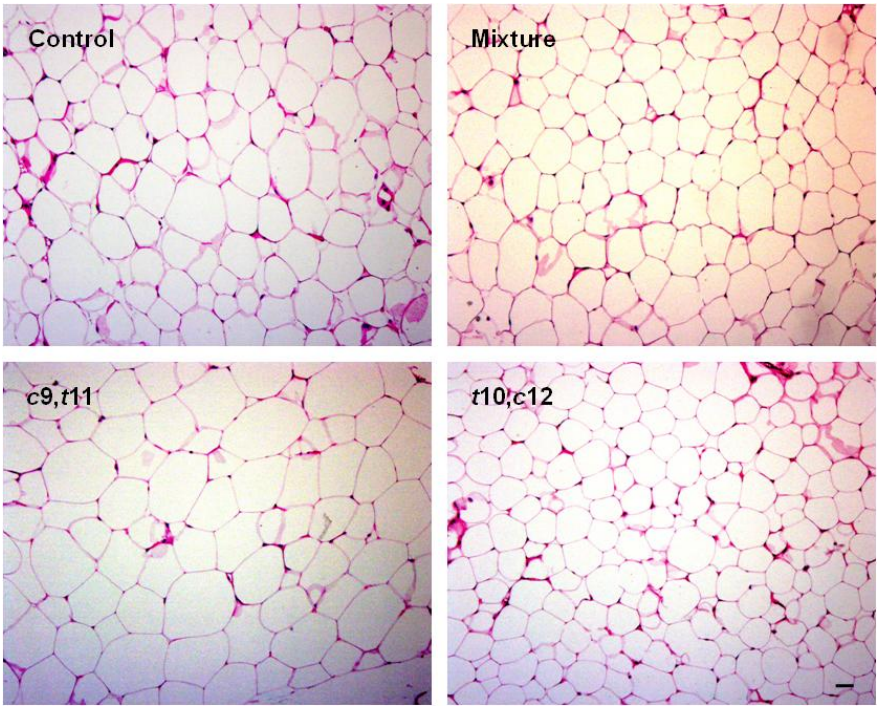

Figure 3.1 Histological images of retroperitoneal fat depot (scale bar: 100 µm).

The lack of interaction between diet and fat ($P>0.05$) enabled the presentation of the means for each factor (diet or fat), regardless of the other, as shown in Table 3.3. Histometrical results showed that diet and fat depot had a significant effect on adipocyte size and number in an area of 360×10^3 μm^2 ($P<0.05$). The absence of interaction between the two factors ($P>0.05$) suggests that different CLA diets had a similar impact on the two fat depots studied. Diet supplementation with the *c*9,*t*11 CLA isomer increased adipocyte size (3721 μm^2, $P<0.05$), with a consequent decrease in the number of adipocytes *per* unit of area (108, $P<0.05$). The number of adipocytes *per* unit of area was higher in animals receiving the CLA mixture (132, $P<0.05$) and the *t*10,*c*12 CLA isomer (133, $P<0.05$), when compared to the *c*9,*t*11 CLA isomer group. The type of adipose tissue analysed also affected the two histometrically studied parameters, with epididymal fat depot showing the lowest adipocyte

area (3055 μm^2, P<0.05) and the highest adipocyte number (131, P<0.05). Retroperitoneal fat presented adipocytes with an area of 3319 μm^2 and a count of 117 adipocytes *per* unit of area. To further explore the above-mentioned histologic differences, the area and number of adipocyte distribution in epididymal and retroperitoneal fat depots were divided *per* classes and represented in histograms (Figure 3.2 and Figure 3.3). The adipocyte area distribution (Figure 3.2) reveals that animals of the *c*9,*t*11 CLA isomer group presented a lower proportion of cells in the 800-3200 μm^2 range (P<0.05) and a higher percentage of cells with an area of more than 5600 μm^2 (P<0.05). No other classes of adipocytes were affected by the three CLA treatments (P>0.05). The fat depot effect was critical in the 1600-3200 μm^2 class, showing that the retroperitoneal pad had a lesser adipocyte proportion compared to the epididymal fat (P<0.05). This fact is balanced by the highest percentages observed in classes with larger adipocytes (>4800 μm^2) for retroperitoneal fat. The adipocyte number distribution (Figure 3.3) is markedly affected by diet in the first class (<80), showing an effect of the *c*9,*t*11 CLA isomer in the reduction on cell number in both fat depots (P<0.05). The fat depot factor influenced, in particular, the classes ranging from 80-110 to 140-170 adipocytes (P<0.05).

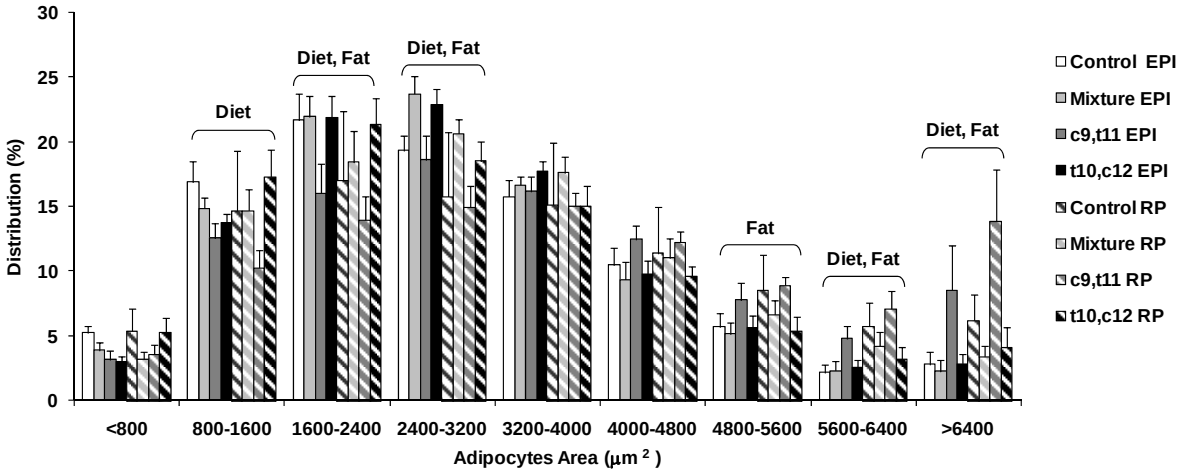

Figure 3.2 Adipocyte area distribution *per* classes for epididymal (EPI; closed bars) and retroperitoneal (RP; hatched bars) fat depots from rats fed CLA diets. Data represent mean values ± SEM. Significant effects of Diet, Fat, and Diet × Fat (according to one-way analysis of variance with Tukey's test, at P<0.05) are noted above each class.

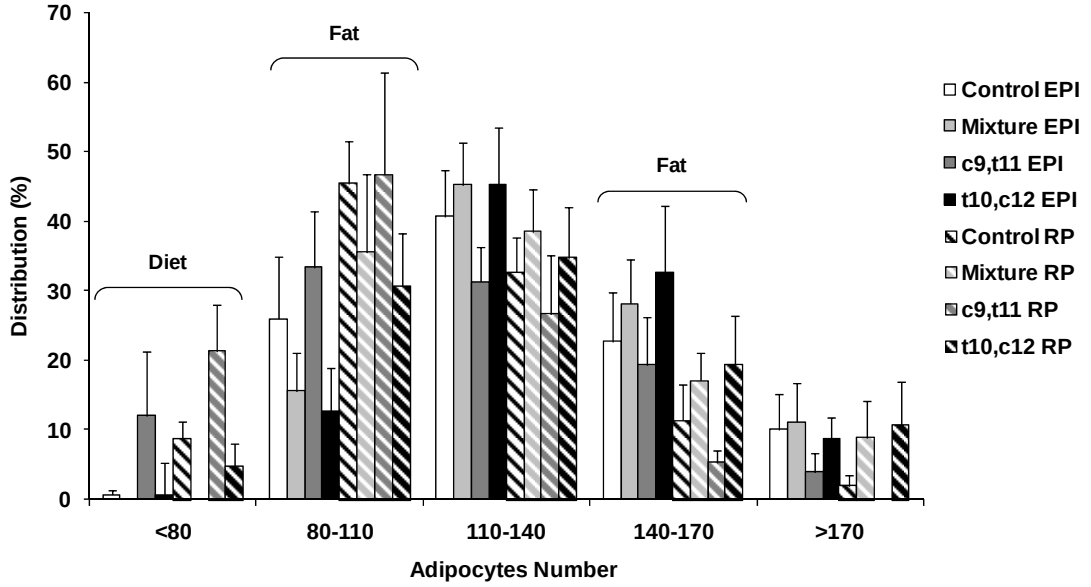

Figure 3.3 Adipocyte number distribution *per* classes for epididymal (EPI; closed bars) and retroperitoneal (RP; hatched bars) fat depots from rats fed CLA diets. Data represent mean values ± SEM. Significant effects of Diet, Fat, and Diet × Fat (according to one-way analysis of variance with Tukey's test, at $P<0.05$) are noted above each class.

The average (110-140) and upper classes (>170) were not influenced by any of the factors under analysis. Diet supplementation with the CLA mixture and the *t*10,*c*12 CLA isomer had no influence on the adipocyte number distribution ($P>0.05$). Finally, in relation to the levels of adipokines in serum, the concentrations of leptin and adiponectin were not significantly affected ($P>0.05$) by any of the dietary CLA treatments (Table 3.2).

3.4. DISCUSSION

Commercial CLA preparations containing *t*10,*c*12 and *c*9,*t*11 isomers in various proportions are attracting consumers' interest owing to the putative fat-lowering effects of these conjugated fatty acids on body composition (Silveira *et al.*, 2007). However, in humans, the effect of dietary CLA on body fat is still far from well characterised, and some conflicting data have been reported (Evans *et al.*, 2002; Brown & McIntosh, 2003; Silveira *et al.*, 2007). The main goal of this study was to evaluate, histometrically, the isomer-specific influence of CLA on body fat of Wistar rats and to compare the effects of individual isomers with a CLA mixture to identify possible interactions between isomers. Diets were formulated to contain 5% palm oil to increase the percentage of saturated fatty acids, which are typical in today's Western diets. This procedure allowed the potential elucidation of CLA involvement in human metabolism when fed with saturated diets. Histologic data presented in this work revealed that dietary *c*9,*t*11 CLA isomer supplementation significantly increased adipocyte

size with a consequent reduction in the number of adipocytes *per* area. Available literature analysing the anti-adipogenic effects of CLA *in vivo*, evaluating the role of individual isomers in adipocyte morphology, is scarce (Azain *et al.*, 2000; Poulos *et al.*, 2001; Corino *et al.*, 2005; Simón *et al.*, 2005; Noto *et al.*, 2007). At a 0.5% CLA supplementation, no effects were observed on growth rate, feed intake, and liver weight in rats (Azain *et al.*, 2000). However, in contrast to our findings, retroperitoneal and epididymal fat depots were indeed diminished owing to smaller adipocyte size rather than to reduced cell number (Azain *et al.*, 2000). At weaning, rats fed 0.5% dietary CLA led to fewer large adipocytes with an increased number of smaller fat cells (Poulos *et al.*, 2001). Poulos *et al.* (2001) proposed that CLA promotes an early differentiation of adipocytes, thus limiting their size and the capacity for triacylglycerol storage, which results in a decrease in body fat. In the present study, neither of the CLA supplemented groups showed a tendency for this type of physiologic response. Despite the increase in adipocyte size and serum triacylglycerols observed in the *c*9,*t*11 CLA isomer group, this dietary CLA isomer had no significant effect on final body and fat depot weights. It has been suggested that retroperitoneal fat is more responsive than epididymal and parametrial fats to different concentrations of CLA (Azain *et al.*, 2000). A dose-dependent effect of a dietary CLA mixture on many adipose depots was well exploited by DeLany *et al.* (1999). That study concluded that retroperitoneal fat was more sensitive than inguinal, epididymal, and mesenteric fat depots to dietary CLA. In the present work, CLA diets promoted a similar response in epididymal and retroperitoneal fat depots. Nevertheless, adipocytes were statistically larger in retroperitoneal pad when compared to the epididymal fat cells. This could reveal different metabolic dynamics of these two fats.

Adipocytes work to control appetite and fatty acid oxidation in peripheral tissues, such as liver and skeletal muscle, through leptin and adiponectin secretion, respectively (Whitehead *et al.*, 2006). A positive correlation was previously reported between serum leptin and adipose tissue weight (Akahoshi *et al.*, 2002; Yamasaki *et al.*, 2003). Fruebis *et al.* (2001) concluded that adiponectin contributes to weight loss in mice by its above-mentioned actions. In addition, CLA has been reported to modulate adipokine metabolism. In this study, higher adipocyte size was observed in the *c*9,*t*11 CLA isomer dietary treatment, which suggests that variations in serum leptin and adiponectin concentrations could be expected. It has been generally accepted that circulating leptin levels correlate with adipose mass (Noto *et al.*, 2007). However, some reports observed a marked decrease in leptin concentrations from large, dysfunctional adipocytes (Guo *et al.*, 2004). Adipokine concentrations in serum were similar across the four groups, even in the *c*9,*t*11 CLA isomer dietary treatment, which may

be reasonably justified by the lack of variation in final body and fat depot weights. Two important limitations of this study should be acknowledged. Primarily, the lack of experimental groups fed an unsaturated based diet (with corn or sunflower oils, for instance), which could have clarified how different types of fat can interfere and modulate CLA metabolic responses. In fact, a complex interaction between dietary manipulations (protein type and fat level) and the body fat reducing action of CLA has already been reported (Hargrave *et al.*, 2004; Akahoshi *et al.*, 2005). Secondly, the relative leanness of the Wistar model may be responsible for the absence of significant effects of dietary CLA on rat's body weight, as reported previously by Azain *et al.* (2000).

3.5. CONCLUSIONS

In conclusion, this study demonstrates that supplementation of a palm oil based diet in Wistar rats with the *c*9,*t*11 CLA isomer, in contrast to the *t*10,*c*12 CLA isomer and the mixture of both isomers, affects the histometrical pattern of white adipose depots, although leading to minor variations in serum lipid profiles and adipokines. Under these experimental conditions, the *c*9,*t*11 CLA isomer increases adipocyte size, followed by a decrease in adipocyte number *per* area, through biochemical pathways that remain presently unclear. The CLA mixture does not affect body fat composition, adipocyte size, and number. This suggests that somehow the *t*10,*c*12 CLA isomer, in these conditions, counterbalances the effects of the *c*9,*t*11 CLA isomer. Finally, further research is needed to clarify the potential anti-obesity effects of CLA isomers and their underlying molecular pathways in different fat based diets.

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CHAPTER 4 Effect of dietary conjugated linoleic acid isomers on water and glycerol permeability of kidney membranes

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Abstract

Conjugated linoleic acid (CLA) refers to a group of positional and geometrical isomers of linoleic acid in which the double bonds are conjugated. Dietary CLA has been associated with various health benefits although details of its molecular mode of action remain elusive. The effect of CLA supplemented to palm oil based diets in Wistar rats, as a mixture of both or isolated *c*9,*t*11 and *t*10,*c*12 isomers, was examined on water and glycerol membrane permeability of kidney proximal tubule. Although water permeability was unaltered, an increase in glycerol permeability was obtained for the group supplemented with CLA mixture, even though the activation energy for glycerol permeation remained high. This effect was correlated with an increased CLA isomeric membrane incorporation for the same dietary group. These results suggest that diet supplementation with CLA mixture, in contrast to its individual isomers, may enhance membrane fluidity subsequently raising kidney glycerol reabsorption.

4.1. INTRODUCTION

Conjugated linoleic acid (CLA) refers to a multiplicity of geometrical and positional isomers of linoleic acid with conjugated double bonds. CLA has been associated with some health benefits and, for this reason, has attracted much scientific attention. A growing number of experimental studies using laboratory animals, as well as human and cell culture systems, suggests that *c*9,*t*11 and *t*10,*c*12 CLA isomers may prevent heart disease, diabetes and atherosclerosis, affect weight control and inhibit the growth of various types of cancer (Bhattacharya *et al.*, 2006). The most abundant CLA isomer in ruminant foods is the *c*9,*t*11 (Martins *et al.*, 2007), produced in the rumen by biohydrogenation of dietary C18 PUFA and in the tissues through Δ9-desaturation of 18:1*t*11, although nutritional supplements posses a mixture of equal amounts of *c*9,*t*11 and *t*10,*c*12 isomers. These commercial CLA preparations industrially produced are attracting consumers' interest due to the purported body fat-lowering effects of CLA, coupled to the perception of a 'natural' compound. However, the attempts to replicate these beneficial findings in humans have produced inconsistent results (Salas-Salvado *et al.*, 2006; Plourde *et al.*, 2008). Albeit several candidate mechanisms including alterations in membrane structure and composition, signal transduction, gene expression and immunity have been suggested as future research directions (Field & Schley, 2004), the metabolic pathways by which CLA isomers elicit their effects on body composition remain largely unknown.

In the kidney, at least seven aquaporins are expressed at different sites along the nephron. The orthodox aquaporin-1 (AQP1) is extremely abundant in the proximal tubule and descending limb where it appears to be the main site for proximal tubule water reabsorption. The other aquaporin isoform abundantly expressed at proximal tubule brush border membrane is AQP7, an aquaglyceroporin which was also found in other tissues like testis and adipocytes (Ishibashi *et al.*, 1997; Kuriyama *et al.*, 1997). In the kidney, AQP7 plays a minor role in water transport but constitutes a major glycerol-reabsorbing pathway preventing glycerol from being excreted into urine (Sohara *et al.*, 2005). Besides molecular biology approaches that include aquaporin gene deletion and overexpression, evaluation of the osmotic permeability coefficient (P_f) and Arrhenius activation energy (E_a) are among the few experimentally measurable parameters that indicate the contribution of AQPs to water transport, thus playing an important role in the debate on the physiological function of specific AQPs and their regulation. As for glycerol transport, fewer studies have been performed on kidney apical membranes glycerol permeability (P_{gly}). The only reported data

has been taken from measuring the serum and urine glycerol levels in wild-type and AQP7 knockout mice (Sohara *et al.*, 2005). Despite being under attention by the research community during the last decades, no studies have been published addressing CLA isomers effects on kidney water and glycerol reabsorption. In this study, the effect of dietary CLA isomers on both water and glycerol permeabilities of kidney proximal tubule membranes were investigated. The preparation of isolated brush-border membrane vesicles (BBMV) provides the possibility of studying transport processes independently of other cellular events. Moreover, being more resistant and viable than intact cells, vesicles can be prepared with any chosen internal media, expanding the range of experimental conditions. Four groups of Wistar rats were fed saturated diets, formulated to mimic patterns of typical human diets from the Western societies (Edem, 2002) and supplemented or not with *c*9,*t*11 and *t*10,*c*12 CLA isomers in separate or as a mixture. Using purified kidney BBMV preparations, membrane permeabilities P_f and P_{gly} were assessed by stopped-flow light scatter. The E_a for water and glycerol transport was calculated. Additionally, the fatty acid profile of membrane vesicles was determined in order to assess CLA isomers incorporation level into total membrane lipids.

4.2. MATERIALS AND METHODS

4.2.1. Experimental design, animals and diets

The experimental protocol of this study was reviewed by the ethics commission of CIISA/FMV and approved by the Animal Care Committee of the National Veterinary Authority following the appropriated European Union guidelines (N. 86/609/EEC). Wistar male rats (*n*=39, Harlan Interfauna Iberica, S.L., Barcelona, Spain) with an initial body weight of 114.7 ± 0.98 g (mean ± SEM) were acclimatised and fed a standard diet (Harlan Teklad Global Diets©2014) without CLA for 1 week. After this period, rats were allocated to four groups of ten animals each. The standard pellets were enriched with palm, sunflower and CLA oils as described (Lopes *et al.*, 2008). Briefly, a 5% palm oil based diet was supplemented with CLA oils to reach 1% of CLA isomers (0.5% of *c*9,*t*11 and 0.5% of *t*10,*c*12) for the group Mixture, and 0.5% of *c*9,*t*11 or 0.5% of *t*10,*c*12 isomers for groups *c*9,*t*11 and *t*10,*c*12, respectively. A control group with 5% of palm oil and without CLA was included for comparative purposes. The diets were provided *ad libitum* during 8 weeks and, after a 12 h fast, rats were euthanised by decapitation, under light inhalation anesthesia

(isofluorane). The kidneys were excised and washed in 150 mM NaCl, 10 mM Tris–HCl pH 7.4, at 4 ºC, for subsequent preparation of BBMV.

4.2.2. Preparation of brush-border membrane vesicles

BBMV were prepared from rat renal cortex as described Soveral *et al.* (1997a). After kidney decapsulation, the whole process was conducted at 4 ºC in the presence of a single buffer containing 100 mM mannitol, 10 mM Tris–Hepes pH 7.4. Kidney cortexes from each dietary group were pooled to give one final vesicle preparation. Prior to osmotic experiments, vesicles were resuspended and homogenised in the same buffer but where mannitol was replaced by cellobiose (final osmolarity 120 mOsM), a solute shown to be highly impermeant over a larger time scale (Soveral *et al.*, 1997b). The membrane preparations obtained were either immediately used for experiments or stored in liquid nitrogen for later use. Protein content was determined by the Bradford technique (Bradford, 1976), using bovine albumin as standard. All solution osmolarities were determined from freezing point depression on a semi-micro osmometer (Knauer GmbH, Germany). Standards of 100 and 400 mOsM were analysed prior to samples, which were measured in triplicate. Vesicle size of all the membrane preparations was determined in isosmotic conditions by the Quasi-Elastic Light Scattering (QELS) technique (Brookhaven Instruments BI-90). Application of this technique to vesicular size measurements has been published (Perevucnik *et al.*, 1985).

4.2.3. Stopped-flow experiments on water and glycerol permeability

Stopped-flow experiments were performed on a HI-TECH Scientific PQ/SF-53 apparatus, which has a 2 ms dead time, temperature controlled, interfaced with an IBM PC/AT compatible 80386 microcomputer. Experiments were performed at different temperatures. Typically, five runs were usually stored and analysed in each experimental condition. For the measurement of osmotic water permeability, 0.1 ml of vesicles (0.4 mg protein/ml) resuspended in cellobiose buffer was mixed with an equal amount of isoosmotic (120 mOsM) or hyperosmotic (240 mOsM) cellobiose solutions to reach an inwardly directed gradient of solute. The kinetics of vesicle shrinkage were measured from the time course of 90º scattered light intensity at 400 nm until a stable light scatter signal was attained. The osmotic water permeability coefficient was estimated by fitting the light scatter signal to a single exponential curve and using the linear relation between P_f and the exponential time constant k (Van Heeswijk & Van Os, 1986), $P_f = k(V_o/A)(1/V_w(osm_{out})_\infty)$, where V_w is the molar volume of

water, V_o/A is the initial volume to area ratio of the vesicle preparation, and $(osm_{out})_\infty$ is the final medium osmolarity after the applied osmotic gradient. For glycerol permeability, vesicles equilibrated in 120 mOsM cellobiose buffer were confronted to an external solution where the impermeant cellobiose was partially substituted with glycerol (60 mOsM cellobiose, 60 mOsM glycerol, creating an inwardly directed glycerol gradient with no osmotic shock). Glycerol influx in response to its chemical gradient was followed by water influx with subsequent vesicle swelling. Glycerol permeability was calculated as $P_{gly} = k(V_o/A)$, where k is the single exponential time constant fitted to the light scattering time course corresponding to glycerol influx (Dix *et al.*, 1985).

4.2.4. Activation energy calculation

Water and glycerol permeabilities were measured at five different temperatures between 7 ºC and 37 ºC. The Ea of water and of glycerol transport was evaluated from the slope of the Arrhenius plot (lnP_f or lnP_{gly} as a function of 1/T) multiplied by the gas constant R.

4.2.5. Fatty acid profile

The fatty acid profile of BBMV preparations, resulting from pooled kidney cortexes for each dietary group, was obtained using the transesterification method (Raes *et al.*, 2004). The fatty acid methyl esters were analysed through single injections by gas chromatography (GC) and methyl esters of CLA isomers were individually separated by high performance liquid chromatography (HPLC). The percentage of CLA isomers was calculated from their HPLC areas relative to the area of the main isomer *c*9,*t*11 identified by GC (Rego *et al.*, 2008).

4.2.6. Statistics

Data are expressed as mean ± SD. Values of permeability coefficients were analysed and compared with control using the Student's t test. Differences with $P<0.05$ were considered significant.

4.3. RESULTS

4.3.1. Characterisation of BBMV from kidney proximal tubule

BBMV prepared from rat kidney cortex by differential centrifugation showed an enrichment in enzyme specific activity (BBMV/crude homogenate) of the apical markers leucine-aminopeptidase (Kramers & Robinson, 1979) and alkaline phosphatase (Quamme, 1990), as well as the basolateral markers Na^+/K^+ATPase (Quigley & Gotterer, 1969) and K^+ stimulated phosphatase (Murer *et al.*, 1976), assayed as described, of 13.2 ± 1.7, 9.6 ± 1.5, 1.1 ± 0.04 and 0.4 ± 0.05 (*n*=20), respectively. Vesicle size of all prepared batches of vesicles determined by QELS revealed homogeneous populations, showing unimodal distributions with a mean diameter of 415 ± 35 nm (*n*=26). These results assure purified and homogeneous BBMV preparations.

4.3.2. BBMV permeabilities to water and glycerol

To assess the effect of dietary CLA isomers on water membrane permeability, we measured the P_f of purified BBMV. The osmotic challenges were performed at different temperatures using the stopped-flow technique.

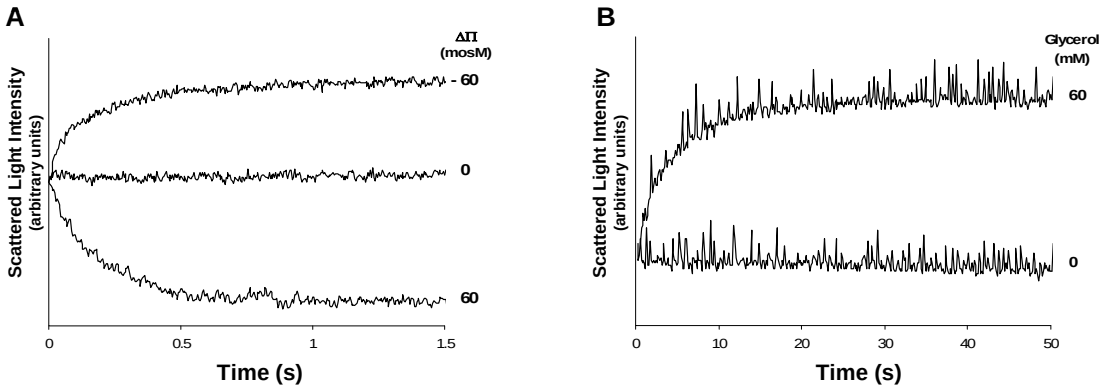

Figure 4.1 Representative plots of brush border membrane permeability to water (A) (osmotic shocks of -60, 0 and 60 mOsM cellobiose gradients) and glycerol (B) (isosmotic shock, 60 mOsM glycerol gradient), by stopped-flow light scattering.

Figure 4.1A shows a typical time course of volume change induced by osmotic shocks of -60, 0 and 60 mOsM cellobiose, carried out with the control group membrane vesicles at 23 ºC. BBMV were osmotically responsive when confronted to hypo- and hyper-osmotic shocks of impermeant solute, as can be seen by the light scatter traces reflecting volume changes. Averaged P_f was $(13.86 \pm 2.11)\times10^{-3}$ cm.s^{-1}. Glycerol transport was studied in membrane

vesicles prepared from control and CLA groups submitted to an inward 60 mOsM glycerol gradient in isoosmotic conditions, in order to avoid initial water movements. As glycerol enters, vesicles progressively swell till they reach an equilibrium volume, as shown in Figure 4.1B, and the rate constants of glycerol influx were used for P_{gly} evaluation. Averaged P_{gly} for the control group was $(2.55 \pm 0.63)\times10^{-6}$ cm.s^{-1}. Figure 4.2 shows the permeability results obtained for the dietary groups at 23 ºC, for water (panel A) and glycerol (panel B).

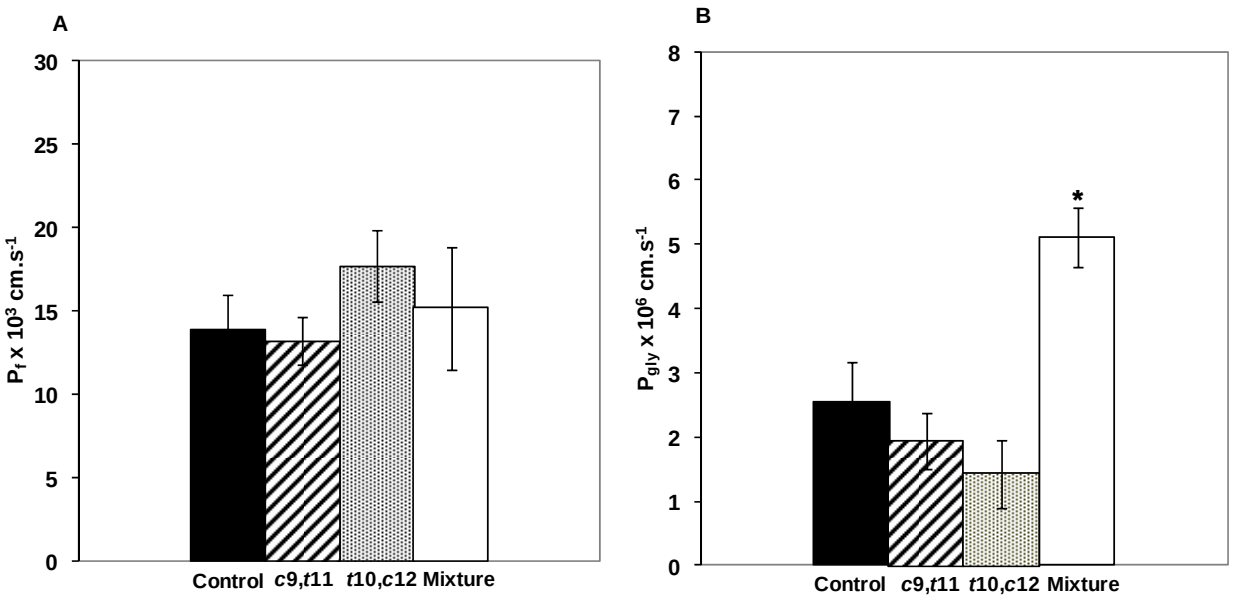

Figure 4.2 Water (A) and glycerol (B) permeability coefficients at 23 ºC for the different membrane vesicles. Values are mean ± SD of triplicates of at least three independent experiments (n=9). * Different from control, P<0.05.

It can be observed that the values of osmotic water permeability P_f are within the same range with no significant differences among groups (P>0.05, Figure 4.2A). As for glycerol, a relevant increase in P_{gly} to roughly twofold the control (P<0.05) was obtained for the mixed isomers (group Mixture) at all temperatures tested. However, *c*9,*t*11 and *t*10,*c*12 CLA isomers did not show any significant change compared with the control (P>0.05, Figure 4.2B). Figure 4.3 depicts the E_a values for both water and glycerol transport. The E_a for glycerol transport was similar among groups (P>0.05) being always very high, above 17.6 kcal.mol^{-1} (73.7 kJ.mol^{-1}). Conversely, the E_a obtained for water transport was low for any group tested, between 4.4 and 4.5 kcal.mol^{-1} (18.4 and 18.8 kJ.mol^{-1}) but still not different among groups (P>0.05).

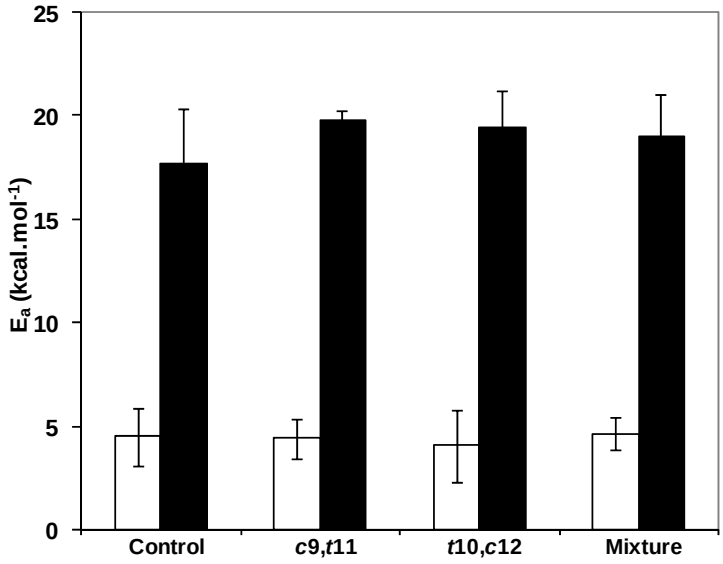

Figure 4.3 Activation energy of water (empty bars) and glycerol (full bars) for the dietary groups tested. Values are mean ± SD (n=3). No significant differences between the dietary CLA groups and control within each type of permeability were detected (P>0.05).

4.3.3. Incorporation of CLA in the BBMV

The fatty acid composition of BBMV from the four dietary groups was particularly different for CLA isomers percentages, as shown in Table 4.1. The deposition of total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) showed only small differences between the four dietary groups. Briefly, groups *t*10,*c*12 and Mixture presented higher percentages of SFA, mainly due to incorporation of 18:0 and 22:0 fatty acids. Group *t*10,*c*12 showed reduced MUFA due to lower levels of 16:1*c*9, 18:1*c*9 and 18:1*c*11 fatty acids. Group *c*9,*t*11 presented a higher percentage of total PUFA, whereby 20:4*n*-6 is the most responsible for this increase. Regarding the CLA isomeric profile, major differences were observed between the four dietary groups. As expected, CLA was not detected in the control group. The diet for both groups *c*9,*t*11 and *t*10,*c*12 contained the same amount of the respective CLA isomer, and the diet of group Mixture contained the same amount of both isomers, as referred in the Methods section. However, the incorporation profile in the membranes was different in the three supplemented groups. Group *c*9,*t*11 achieved 0.181% of *c*9,*t*11 CLA isomer, group *t*10,*c*12 showed only 0.029% of *t*10,*c*12 CLA isomer, while group Mixture presented 0.220% and 0.105% of *c*9,*t*11 and *t*10,*c*12, respectively. These differences suggest a poor incorporation in the membrane of *t*10,*c*12 CLA when the diet contains the isolated isomer (group *t*10,*c*12). The presence in the diet of both

isomers (group Mixture) amplifies both their inclusion in the membrane, with a larger than threefold increase in *t*10,*c*12 CLA isomer.

Table 4.1 Fatty acid profile (% fatty acids) of membrane vesicles.

	Control	***c*9,*t*11**	***t*10,*c*12**	**Mixture**
16:0	21.40	20.36	21.75	20.79
17:0	0.22	0.23	0.24	0.26
18:0	19.30	20.86	21.99	22.41
20:0	0.17	0.08	0.08	0.11
22:0	0.09	0.19	0.33	0.43
24:0	0.00	0.10	0.08	0.18
Σ SFA	**41.18**	**41.83**	**44.47**	**44.18**
16:1*c*9	0.98	0.35	0.26	0.41
18:1*c*9	6.79	6.65	6.57	6.76
18:1*c*11	3.05	2.05	1.73	1.90
20:1*c*11	0.11	0.10	0.10	0.17
Σ MUFA	**10.93**	**9.15**	**8.66**	**9.25**
18:2*n*-6	11.74	10.69	11.78	11.94
18:3*n*-3	0.14	0.05	0.05	0.15
20:2*n*-6	0.27	0.30	0.30	0.16
20:3*n*-3	0.00	0.07	0.09	0.23
20:3*n*-6	1.22	0.61	0.64	0.64
20:4*n*-6	31.18	34.24	31.15	30.07
20:5*n*-3	0.12	0.07	0.08	0.12
22:4*n*-6	0.81	0.65	0.72	0.59
22:5*n*-3	0.25	0.23	0.27	0.45
22:6*n*-3	2.16	1.89	1.72	1.75
Σ PUFA	**47.89**	**48.81**	**46.81**	**46.10**
18:2*t*7,*c*9	n.d.	0.011	0.011	0.038
18:2*c*9,*t*11	n.d.	0.181	0.003	0.220
18:2*t*10,*c*12	n.d.	0.002	0.029	0.105
18:2*c*/*t*11,13	n.d.	0.005	0.000	0.016
18:2*c*/*t*12,14	n.d.	0.004	0.003	0.027
Σ C18:2*t*,*t*	n.d.	0.015	0.008	0.065
Σ CLA	**n.d.**	**0.219**	**0.054**	**0.471**

n.d., not detected. Σ SFA, sum of saturated fatty acids; Σ MUFA, sum of monounsaturated fatty acids; Σ PUFA, sum of polyunsaturated fatty acids; Σ CLA, sum of conjugated linoleic acid isomers.

4.4. DISCUSSION

The novelty of this study derives from the description of water and glycerol permeability of kidney proximal tubule membrane vesicles, from rats fed CLA on a diet supplemented with a mixture of *c*9,*t*11 and *t*10,*c*12 isomers, or both in separate. Since commercial CLA is composed by a mixture of two isomers, it is of interest to determine which is responsible for biological activity through possible modulation on cell membrane permeability. The use of

isolated BBMV offers an experimental system that allows the composite permeability of epithelial cells to be split into its component parts and yields more precise information about driving forces of transport in epithelia.

In this study, CLA did not affect water transport through the vesicle membrane, neither as a mixture nor using both isomers in separate. In fact, the low E_a obtained is compatible with channel mediated water fluxes (Farmer & Macey, 1970), independently from the tested group. Regarding the E_a for glycerol, the strong temperature dependence revealed by the high value obtained suggests lipid- rather than channel-mediated glycerol transport (Yang *et al.*, 2006). This result may indicate that the aquaglyceroporin AQP7 was not functional or that its level of expression/incorporation in the brush border membrane was very low, resulting in a main contribution of the lipid pathway to glycerol permeability whichever the tested dietary group. Hence, as no channels accounted for glycerol transport and mainly the lipid pathway was being used, any difference in membrane fluidity may lead to increased bilayer permeability to glycerol. Higher P_{gly} relative to control with no change in E_a was detected for the dietary group fed a mixture of CLA isomers, suggesting an effect on lipid bilayer fluidity rather than on the protein channel. In separate, *c*9,*t*11 and *t*10,*c*12 CLA isomers did not significantly alter the glycerol permeability, but together, they potentiate a significant effect.

The fatty acid profile of membrane vesicles showed only slight differences among dietary groups for the partial sums of SFA, MUFA and PUFA. On the contrary, CLA isomers incorporation into membranes was distinct among all groups. It has been suggested that both major CLA isomers specifically replace the essential fatty acids arachidonic (20:4*n*-6) and linoleic (18:2*n*-6) into membrane phospholipids (Field & Schley, 2004), but our results do not support this finding (Table 4.1). In our work, CLA isomeric distribution in membrane vesicles was not proportional to its correspondent percentages added to the diet. As expected, membranes from the group Mixture fed 1% of combined isomers *c*9,*t*11 and *t*10,*c*12 were the richest in CLA, showing also higher proportions of the minor isomers. It has been reported that incorporation of unsaturated fats into cellular membranes increases membrane fluidity and permeability (Hill *et al.*, 2005; Poulsen *et al.*, 2007). The greatest increase in fluidity occurs with the addition of 2 and 3 double bonds, that allow considerable bending in the fatty acid chain thus inducing a decrease in the average chain length (Stillwell & Wassall, 2003). A recent review compiles results from a battery of biophysical techniques demonstrating that the insertion of docosahexaenoic acid (DHA; 22:4*n*-6) into membranes creates liquid disordered domains containing DHA-phospholipids and depleted in cholesterol, having looser lipid

packing, thereby increasing membrane fluidity (Wassall & Stillwell, 2008). Our results suggest that the *c*9,*t*11 isomer intake promotes a higher incorporation of the *t*10,*c*12 in the membrane, thereby increasing the final CLA amount (group Mixture, Table 4.1.). In addition to total CLA amount, the diversity of isomers found in the mixture group may alter fatty acids arrangement and their packing in the bilayer changing membrane fluidity, thus explaining the observed increase in glycerol permeability. An increase in bilayer permeability has also been reported for liposomes containing CLA isomers (Yin *et al.*, 2006).

4.5. CONCLUSIONS

In conclusion, this study suggests that a palm oil based diet supplemented with a mixture of *c*9,*t*11 and *t*10,*c*12 CLA isomers in Wistar rats, in contrast to the individual isomers, affects the glycerol membrane permeability of kidney proximal tubule, possibly by means of an increase in lipid bilayer fluidity. This fact may be of biological relevance since in the kidney, glycerol can be either metabolised *in situ* or converted to glucose in the liver. Conversely, the data indicates that CLA isomers, isolated or as a mixture, do not affect aquaporin-mediated water transport.

Acknowledgements

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CHAPTER 5 Effect of CLA on fatty acid composition in the liver and muscle of obese Zucker rats fed different saturated fat diets

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Submitted

Abstract

The purpose of this study was to investigate the combined effect of conjugated linoleic acid (CLA) and saturated fats from distinct sources, vegetable and animal, on fatty acid profile in liver and muscle of obese Zucker rats. Thirty-two male rats were randomly assigned to one of four atherogenic diets containing palm oil (P) and ovine fat (O), and supplemented with 1% of CLA (PCLA and OCLA). In CLA-fed rats, the SFA content in the liver increased, whereas the MUFA content decreased. The percentage of PUFA in hepatocytes remained unchanged. In muscle, CLA did not affect SFA but decreased MUFA and PUFA contents. The TFA were enhanced by CLA supplementation. The estimation of Δ9-index16 and Δ9-index18 suggested that CLA inhibited the SCD activity in the liver, and, specially, when supplemented to the ovine fat diet. In liver, long chain fatty acids from *n*-3 series were increased by CLA when supplemented to ovine fat diet. In muscle, CLA diminished *n*-6 long chain fatty acids. In both studied tissues, the *t*10,*c*12 CLA isomer was less incorporated than the *c*9,*t*11 CLA isomer. The ingestion of ovine fat based diets resulted in the incorporation of several ruminant isomers. Overall, this study showed that CLA altered the fatty acid profile and was dependent upon dietary fat source.

5.1. INTRODUCTION

The initial interest in conjugated linoleic acid (CLA) arose in its anticarcinogenic properties (Ha *et al.*, 1987), but many other biological actions of CLA have been studied with a huge focus on their capacity to modify the lipid metabolism and to reduce the body fat mass (Park *et al.*, 2007a). CLA defines a group of isomers derived from linoleic acid with conjugated double bounds. Ruminant food products are rich in CLA, predominantly in the rumenic acid, *c*9,*t*11 CLA isomer, that is produced by bacterial hydrogenation and isomerisation of linoleic acid in the gut of ruminant animals, as well as, via Δ9-desaturation of vaccenic acid (18:1*t*11) in ruminant tissues. The conversion of vaccenic acid to rumenic acid by stearoyl-CoA desaturase (SCD) was also observed in other animal species, such as, mouse (Santora *et al.*, 2000), rat (Lock *et al.*, 2004), pig (Glaser *et al.*, 2002) and human (Turpeinen *et al.*, 2002). Although the majority of dietary CLA in foodstuff is found as rumenic acid, CLA supplements often include the *t*10,*c*12 CLA isomer in equivalent amounts. Either these two isomers, or the *t*10,*c*12 CLA isomer alone, may inhibit the activity of SCD, depending on the animal model used (Park *et al.*, 2000; Lin, *et al.*, 2004). As CLA can be incorporated into membrane phospholipids, it may compete in elongation and desaturation pathways with other unsaturated fatty acids (Banni *et al.*, 1999). Additionally, rumenic acid may use the same *in vivo* metabolic pathway of conversion into long chain fatty acids, as linoleic acid (Sébédio *et al.*, 2001). Consequently, CLA may alter desaturation activities and modulate the fatty acid profile of different tissues (Bretillon *et al.*, 1999).

Today's unhealthy diet and lifestyles promote excessive intakes of saturated fats (WHO, 2003). High fat diets, possessing inappropriate portion of unsaturated fatty acids, may directly increase the prevalence of obesity worldwide (Li *et al.*, 2008). Ruminant edible fats are a major contributor to saturate fat intake (Givens, 2005). In contrast, dietary polyunsaturated fatty acids (PUFA) have been recognised as capable of reducing the accumulation of abdominal adipose tissue and lipids in the liver and serum, and also to alleviate hypertension and type 2 diabetes, through the regulation of many enzymes involved in lipid and glucose metabolisms (Nagao & Yanagita, 2008). A study reported that CLA is more beneficial for control blood lipids and adiposity when supplemented to a diet rich in saturated versus unsaturated fat (Kloss *et al.*, 2005). However, research performed so far has not adequately compared the CLA effects on tissue fatty acid profile when supplemented in diets containing different sources of saturated fats. Moreover, ruminant edible fat is highly saturated and contains numerous isomers of oleic and linoleic acids originated from incomplete rumen

biohydrogenation of these fatty acids. Differential deposition of these isomers in rat tissues have not been yet reported. We hypothesised that CLA supplementation would had different effects on tissue fatty acid composition under distinct saturated fat based diets, from vegetable and ruminant origin. This study was designed to search for differential responses of CLA supplementation on fatty acid profile of liver and muscle from obese Zucker rats fed atherogenic diets with palm oil and ovine fat. As ruminant fat has a very complex fatty acid profile, a detailed characterisation of feed and tissues was conducted using both gas and high performance liquid chromatographies.

5.2. EXPERIMENTAL PROCEDURES

5.2.1. CLA oil and experimental diets

The CLA oil, containing similar proportions of *c*9,*t*11 CLA and *t*10,*c*12 CLA isomers with 80% purity, was a generous gift from PharmaNutrients Inc. (Gurnee, IL, USA). The remaining ingredients, apart from ovine fat, were purchased by Provimi Kliba SA (Kaiseraugst, Switzerland) to manufacture the experimental diets.

The diets were prepared and pelleted by Provimi Kliba, and followed the AIN-93G formulation with some modifications to obtain atherogenic diets. Diets ingredient composition (% feed) was: casein (20.0), dextrose (13.2), sucrose (11.9), corn starch (29.3), cellulose (5.0), vitamin-mixture (0.5), mineral-mixture (2.4), amino acids (0.3), cholesterol (2.0), cholic acide sodium salt (0.5), BHT (0.01). Diets presented a dry matter (DM) concentration of 928 g/kg feed and the following crude composition (g/100 g DM): protein (18.0), fat (15.0), ash (3.5), fibre (3.5), NEFA (50.0). The four experimental diets differed in the fat composition. Two groups received vegetable fat based diets: group P - 11.25% of palm oil plus 3.75% sunflower oil; group PCLA - 11.25% of palm oil plus 2.53% sunflower oil plus 1.22% CLA. The other two groups (O and OCLA) received ovine fat instead of palm oil. Peritoneal ovine fat was collected from lambs raised with forage enriched in seed oils, as described in Jerónimo *et al.* (2009). Thus, the ovine fat used was enriched in TFA derived from rumen biohydrogenation allowing a good contrast with the simple vegetable saturated fat source. The raw ovine fat was melted, and then filtered to subsequent incorporation in the diets. The fatty acid characterisation of the experimental diets is presented in Table 5.1.

5.2.2. Animals

Thirty-two male obese Zucker rats were obtained from Harlan Interfauna Iberia (Barcelona, Spain), housed individually upon arrival and maintained on a 12 h light:dark cycle at 22 ± 2 ºC of temperature. After an adaptation period of one week, the animals were divided into four groups with eight rats each and fed semi-purified atherogenic diets during 13-14 weeks. Animals had free access to water and food. Body weight and feed intake were monitored twice a week. At the end of the experimental assay, rats were killed by decapitation, under light isofluorane anaesthesia. Following blood collection, all organs were dissected, weighed and stored at -80 ºC for further analyses. The experimental protocol of this study was reviewed by the ethics commission of CIISA/FMV and approved by the Animal Care Committee of the National Veterinary Authority (Direcção Geral de Veterinária, Portugal) following the appropriated European Union guidelines (N. 86/609/EEC).

5.2.3. Fatty acid analysis

Liver and *longissimus dorsi* (*LD*) muscle samples were lyophilised (-60 °C and 2.0 hPa) and maintained exsiccated at room temperature. Lipids were extracted using the method of Christie *et al.* (2001), slightly modified by Raes *et al.* (2001). Fatty acids were directly converted to methyl esters by a combined-procedure of methylation, base-catalysis followed by acid-catalysis. Briefly, 1ml of dry toluene was added to 0.25 g of lyophilised samples and fatty acids were methylated with sodium methoxide in anhydrous methanol (0.5 mol/l), for 30 min, followed by hydrogen chloride in methanol (1/1 v/v), for 10 min, at 50 ºC. Fatty acid methyl esters (FAME) were extracted twice with 3 ml of hexane and pooled extracts were evaporated, at 35 ºC under a stream of nitrogen, until a final volume of 2 ml. The resulting FAME were then analysed by gas liquid chromatography using a fused-silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d. × 0.20 mm film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA), equipped with a flame-ionisation detector, as described by Bessa *et al.* (2007). In order to obtain a detailed profile of conjugated isomers of linoleic acid, CLA isomers were individually separated by triple silver-ion columns in series (ChromSpher 5 Lipids, 250 mm × 4.6 mm i.d., 5 μm particle size, Chrompack, Bridgewater, NJ, USA), using a high performance liquid chromatography system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, USA) equipped with an autosampler and a diode array detector (DAD) adjusted to 233 nm, according to the procedure reported previously (Rego *et al.*, 2008). The identification of individual CLA isomers was achieved by comparison of their

retention times with commercial and prepared standards, as well as with values published in the literature. Fatty acid composition was expressed as g/100 g of total fatty acids identified. The amounts of CLA isomers were calculated from their Ag^{+}-HPLC areas relative to the area of the main isomer *c*9,*t*11 CLA identified by GC (which comprises both *t*7,*c*9 and *t*8,*c*10 CLA isomers) as described by Rego *et al.* (2008). Diets followed the same analytical procedure as liver and muscle to obtain the fatty acid profile (Table 5.1).

5.2.4. Statistics

The statistical analysis was performed with the general linear model (GLM) procedure of Statistical Analysis System, version 9.1 (SAS Institute, Cary, NC, USA). The effects of CLA (with or without 1% CLA) and fat source (vegetable or animal) were analysed as a 2 × 2 factorial arrangement with main effects and its interaction included in the model. When the effects were significant, differences between groups were analysed by Tukey´s *post-hoc* test. Statistical significance was set-up at the $P<0.05$ level.

5.3. RESULTS

5.3.1. Liver fatty acid composition

Livers from rats fed CLA contained less total FAME than rats fed no CLA (Table 5.2). In all groups, the major fatty acids in the liver were 16:0, 16:1*c*9, 18:1*c*9 and 18:2*n*-6. As expected, the fat source effect was detected in all fatty acids (excluding the 20:5*n*-3) according to the fatty acid profiles determined in the diets (Table 5.1). The total saturated fatty acids (SFA) content in liver lipids was significantly increased in rats fed CLA. Palm oil fed groups presented more SFA and PUFA contents than ovine fat fed groups, which reflect the fatty acid composition of diets. CLA decreased monounsaturated fatty acids (MUFA) content in both PCLA and OCLA groups, but more effectively in the ovine fat group, being 16:1*c*9, 18:1*c*9 and Σi-18:1 isomers the main responsible for these differences. Despite the sum of PUFA was not affected by CLA inclusion in the diet, some PUFA were. Contents of 18:2*n*-6, Σnc-18:2, 18:3*n*-6, 20:5*n*-3 decreased whereas 20:4*n*-6 increased due to CLA supplementation. As expected, rats fed CLA had significantly more total CLA content (~1.5 g/100 g fatty acids). CLA supplementation increased the content of *n*-3 PUFA sum in animals fed ovine fat diet, mainly due to the 18:3*n*-3, 22:5*n*-3 and 22:6*n*-3.

Table 5.1 Fatty acid composition (g/100 g total FAME) of experimental diets.

	P	**PCLA**	**O**	**OCLA**
Total FAME	12.3	10.5	12.0	12.6
12:0	0.16	0.18	0.09	0.09
14:0	0.92	1.00	1.35	1.34
15:0	0.05	0.06	0.32	0.32
16:0	35.4	37.5	12.6	12.3
16:1*c*7	0.03	0.03	0.24	0.24
16:1*c*9	0.20	0.14	0.57	0.58
17:1*c*9	0.03	0.02	0.15	0.13
18:0	4.23	4.08	21.0	21.5
18:1*c*9	34.9	32.3	22.3	21.0
18:2*n*-6	20.0	16.0	15.7	12.2
18:3*n*-3	0.13	0.11	1.23	1.24
20:0	0.34	0.31	0.22	0.22
20:2*n*-6	0.01	n.d.	0.02	0.03
22:0	0.22	0.15	0.19	0.15
24:0	0.16	0.12	0.10	0.08
Σ i-18:1 isomers	**1.90**	**1.49**	**15.35**	**15.53**
*t6/t7/t8**	0.07	0.07	0.60	0.61
*t*9	0.09	0.09	0.51	0.53
*t*10	0.09	0.08	0.94	0.83
*t*11	0.06	0.06	9.22	9.42
*t*12	n.d.	n.d.	1.07	1.13
*c*11	1.41	1.11	1.09	1.05
*c*12	0.14	0.04	1.13	1.18
*c*13	0.02	0.03	0.16	0.14
*t*16/*c*14	0.02	0.01	0.44	0.45
*c*15	n.d.	n.d.	0.19	0.19
18:2 *n*-6 isomers				
Σ *non-conjugated*	**0.74**	**0.71**	**2.95**	**3.15**
*t*9,*t*12	n.d.	n.d.	0.44	0.47
*t*8,*c*12/*c*9,*t*12†	n.d.	n.d.	0.29	0.41
*t*8,*c*13/*c*9,*t*13†	0.38	0.36	0.18	0.18
*t*9,*c*12	0.36	0.35	0.13	0.14
*t*11,*c*15	n.d.	n.d.	1.59	1.62
*c*9,*c*15	n.d.	n.d.	0.18	0.19
*c*12,*c*15	n.d.	n.d.	0.14	0.14
Σ *conjugated*	**0.06**	**5.26**	**1.93**	**6.50**
*t*12,*t*14	n.d.	n.d.	0.004	0.044
*t*11,*t*13	n.d.	n.d.	0.114	0.120
*t*10,*t*12	0.015	0.078	0.031	0.085
*t*9,*t*11	0.017	0.077	0.073	0.126
*t*8,*t*10	0.002	0.048	0.010	0.011
*t*7,*t*9	<0.001	0.001	0.006	0.005
c/*t*12,14†	n.d.	n.d.	0.021	0.024
*t*11,*c*13	0.007	n.d.	0.414	0.456
*c*11,*t*13	n.d.	n.d.	n.d.	n.d.
*t*10,*c*12	n.d.	2.48	n.d.	2.14
*c*9,*t*11	0.019	2.55	1.2	3.41
*t*8,*c*10	0.003	0.028	0.033	0.054
*t*7,*c*9	<0.001	0.002	0.042	0.048

Experimental diets: P=palm oil; PCLA= palm oil + 1% CLA; O=ovine fat; OCLA= ovine fat + 1% CLA. † these fatty acids co-elute.

But, in the palm oil fat diets, *n*-3 PUFA did not change. CLA did not affect *n*-6 PUFA sum but palm oil groups were richer in *n*-6 PUFA than ovine fat groups, mainly due to 18:2*n*-6. The TFA sum was enhanced by CLA supplementation. About factors interaction, the deposition of some fatty acids were affected by CLA effects depending on the diet based fat source. The 16:1*c*9, 18:3*n*-3, 20:2*n*-6, 22:5*n*-6 and 22:6*n*-3 did not show differences between P and PCLA groups, but were increased in OCLA comparing to O group. The 17:1*c*9 only experienced a decrease in OCLA group comparing to O group. CLA increased 18:0 levels in both fat sources but in a more extent way in OCLA group. CLA decreased the 22:4*n*-6 concentration when supplemented to palm oil diets. CLA supplementation did not affect the following fatty acids: 14:0, 15:0, 16:0*c*7, 20:0, 20:3*n*-6 and 22:5*n*-6. The Δ9-index16 was higher in OCLA than in O group. In both fats, CLA increased the Δ9-index18 ratio but more markedly in OCLA group.

The detailed composition of oleic and linoleic isomers in the liver are presented in Table 5.3. CLA supplementation increased the concentrations of 18:1*t*10, *t*12 and *c*15, but decreased 18:1*c*11 levels. The 18:1*t*11 only increased in OCLA group. Among non-conjugated 18:2 isomers, CLA diminished the *t*8,*c*12/*c*9,*t*12, *t*11,*c*15 and *c*9,*c*15 isomers. Concentration of most of the CLA isomers increased with CLA supplementation, although *c*/*t*12,14, *t*11,*c*13, *c*11,*t*13 and *t*7,*c*9 did not, and *t*12,*t*14, *t*11,*t*13 increased only in ovine fat fed groups. In all dietary groups, the major CLA isomer found in the liver was c9,t11 isomer. The extent of accumulation was lower for the *t*10,*c*12 than for *c*9,*t*11 CLA isomer.

Table 5.2 Effects of CLA supplementation and fat source on total fatty acid content (g/100 g liver) and composition (g/100 g total FAME) in the liver of obese Zucker rats.

						Significance level		
	P	**PCLA**	**O**	**OCLA**	**SEM**	**CLA**	**Fat**	**CLA × Fat**
Total FAME	16.7	14.2	18.1	14.9	0.888	**	ns	ns
14:0	0.68	0.72	0.86	0.77	0.033	ns	**	ns
15:0	0.17	0.18	0.24	0.24	0.011	ns	***	ns
16:0	18.7	19.9	16.5	18.1	0.334	***	***	ns
16:1c7	0.54	0.56	0.61	0.58	0.019	ns	*	ns
16:1c9	9.60^{a}	9.24^{a}	9.80^{a}	7.37^{b}	0.306	***	*	**
17:1c9	0.27^{c}	0.27^{c}	0.66^{a}	0.60^{b}	0.011	**	***	*
18:0	2.60^{d}	3.56^{b}	3.10^{c}	4.49^{a}	0.100	***	***	*
18:1c9	39.2	37.4	36.7	35.4	0.283	***	***	ns
Σ i-18:1	8.96	8.53	10.54	9.76	0.005	**	***	ns
18:2*n*-6	9.64	8.36	7.89	7.14	0.219	***	***	ns
Σ nc-18:2	0.50^{c}	0.49^{c}	1.70^{a}	1.49^{b}	0.028	***	***	**
Σ CLA	0.06^{d}	1.49^{c}	3.04^{b}	4.53^{a}	0.052	***	***	*
18:3*n*-3	0.09^{c}	0.08^{c}	0.36^{b}	0.61^{a}	0.013	***	***	***
18:3*n*-6	0.21	0.18	0.15	0.12	0.010	**	***	ns
20:0	0.04	0.04	0.06	0.06	0.003	ns	***	ns
20:2*n*-6	0.29^{a}	0.29^{a}	0.21^{b}	0.28^{a}	0.012	**	***	**
20:3*n*-6	0.84	0.71	0.42	0.45	0.044	ns	***	ns
20:4*n*-6	3.80	4.42	2.85	3.46	0.116	***	***	ns
20:5*n*-3	0.08	0.06	0.08	0.06	0.005	**	ns	ns
22:4*n*-6	0.70^{a}	0.52^{b}	0.25^{c}	0.24^{c}	0.029	**	***	*
22:5*n*-3	0.08^{c}	0.08^{c}	0.19^{b}	0.27^{a}	0.008	***	***	***
22:5*n*-6	0.55	0.54	0.16	0.19	0.021	ns	***	ns
22:6*n*-3	0.27^{c}	0.30^{c}	0.58^{b}	0.80^{a}	0.023	***	***	***
Σ unidentified	2.13	2.10	2.99	2.99	0.064	**	***	ns
Σ SFA	22.2	24.4	20.8	23.7	0.344	***	**	ns
Σ MUFA	58.6^{a}	56.0^{b}	58.3^{b}	53.7^{c}	0.343	***	***	**
Σ PUFA	17.1	17.5	17.9	19.6	0.363	ns	***	ns
Σ *n*-3	0.52^{c}	0.52^{c}	1.21^{b}	1.74^{a}	0.037	***	***	***
Σ *n*-6	16.0	15.0	11.9	11.9	0.335	ns	***	ns
Σ TFA	0.79	1.00	3.82	4.19	0.070	***	***	ns
Δ9-index16	0.66^{b}	0.68^{ab}	0.63^{c}	0.71^{a}	0.008	***	ns	**
Δ9-index18	0.06^{d}	0.09^{b}	0.08^{c}	0.11^{a}	0.002	***	***	*

Data are mean ± SEM for n=8 rats *per* group; Significance level: not significant (ns), $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; means in the same row with different superscripts are significantly different ($P<0.05$). Experimental diets: P=palm oil; PCLA= palm oil + 1% CLA; O=ovine fat; OCLA= ovine fat + 1% CLA; nc, non-conjugated; CLA, conjugated linoleic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, *trans* fatty acids.

Σ i-18:1=18:1*t*6/*t*7/*t*8, *t*9, *t*10, *t*11, *t*12, *c*11, *c*12, *c*13, *t*6/*c*14, *c*15; Σ nc-18:2=18:2*t*9,*t*12, *t*8,*c*12/*c*9,*t*12, *t*8,*c*13/*c*9,*t*13, *t*9,*c*12, *t*11,*c*15, *c*9,*c*15, *c*12,*c*15; Σ CLA=*t*12,*t*14, *t*11,*t*13, *t*10,*t*12, *t*9,*t*11, *t*8,*t*10, *t*7,*t*9, *c*/*t*12,14, *t*11,*c*13, *c*11,*t*13, *t*10,*c*12, *c*9,*t*11, *t*8,*c*10, *t*7,*c*9; Σ SFA=14:0, 15:0, 16:0, 18:0, 20:0; Σ MUFA= 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, Σ i-18:1; Σ PUFA= 18:2*n*-6, Σ nc-18:2, Σ CLA, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-6, 22:5*n*-3, 22:6*n*-3; Σ *n*-3= 18:3*n*-3, 20:5*n*-3, 22:5*n*-3, 22:6*n*-3; Σ *n*-6= 18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 22:4*n*-6, 22:5*n*-6; Σ TFA= 18:1*t*6/*t*7/*t*8, *t*9, *t*10, *t*11, *t*12, *t*6/*c*14, 18:2*t*9,*t*12, *t*8,*c*12/*c*9,*t*12, *t*8,*c*13/*c*9,*t*13, *t*9,*c*12; Δ9-index16 = 16:0/(16:0 + 16:1*c*9); Δ9-index18 = 18:0/(18:0 + 18:1*c*9).

Table 5.3 Effects of CLA supplementation and fat source on oleic and linoleic isomers (g/100 g total FAME) in the liver of obese Zucker rats.

						Significance level		
	P	**PCLA**	**O**	**OCLA**	**SEM**	**CLA**	**Fat**	**CLA × Fat**
18:1 isomers								
*t*6/*t*7/*t*8†	0.03	0.03	0.12	0.11	0.003	ns	***	ns
*t*9	0.08	0.07	0.25	0.25	0.005	ns	***	ns
*t*10	0.05	0.24	0.36	0.52	0.022	***	***	ns
*t*11	0.02^{c}	0.04^{c}	1.12^{b}	1.45^{a}	0.027	***	***	***
*t*12	0.11	0.13	0.34	0.36	0.009	*	***	ns
*c*11	8.45^{a}	7.76^{b}	7.08^{c}	5.78^{d}	0.135	***	***	*
*c*12	n.d.	n.d.	0.73^{a}	0.69^{b}	0.007	**	***	**
*c*13	0.22	0.26	0.28	0.27	0.018	ns	*	ns
*t*16/*c*14†	n.d.	n.d.	0.16	0.19	0.011	ns	***	ns
*c*15	n.d.	n.d.	0.10^{b}	0.14^{a}	0.008	*	***	*
18:2 *n*-6 isomers								
non-conjugated								
*t*9,*t*12	0.02	0.02	0.04	0.04	0.005	ns	**	ns
*t*8,*c*12/*c*9,*t*12†	n.d.	n.d.	0.54^{a}	0.50^{b}	0.01	ns	***	*
*t*8,*c*13/*c*9,*t*13†	0.13^{b}	0.16^{b}	0.29^{a}	0.27^{a}	0.006	ns	***	**
*t*9,*c*12	0.35	0.31	0.20	0.14	0.011	***	***	ns
*t*11,*c*15	n.d.	n.d.	0.40^{a}	0.36^{b}	0.008	*	***	*
*c*9,*c*15	n.d.	n.d.	0.19^{a}	0.15^{b}	0.004	***	***	***
*c*12,*c*15	n.d.	n.d.	0.04	0.03	0.001	ns	***	ns
conjugated								
*t*12,*t*14	n.d.	n.d.	0.006^{b}	0.008^{a}	<0.001	**	***	**
*t*11,*t*13	n.d.	n.d.	0.011^{b}	0.013^{a}	<0.001	*	***	*
*t*10,*t*12	0.002	0.036	0.006	0.044	0.002	***	*	ns
*t*9,*t*11	0.005	0.038	0.066	0.092	0.003	***	***	ns
*t*8,*t*10	0.003^{b}	0.015^{a}	0.006^{b}	0.011^{a}	0.001	***	ns	*
*t*7,*t*9	0.003^{c}	0.017^{b}	0.022^{ab}	0.026^{a}	0.002	***	***	*
c/*t*12,14†	n.d.	n.d.	n.d.	n.d.	-	-	-	-
*t*11,*c*13	<0.001	n.d.	0.042	0.045	0.004	ns	***	ns
*c*11,*t*13	n.d.	n.d.	n.d.	n.d.	-	-	-	-
*t*10,*c*12	n.d.	0.205	0.004	0.273	0.008	***	***	ns
*c*9,*t*11	0.047	1.10	2.81	3.86	0.048	***	***	ns
*t*8,*c*10	0.003^{c}	0.076^{b}	n.d.	0.108^{a}	0.004	***	***	***
*t*7,*c*9	n.d.	n.d.	0.069	0.053	0.005	ns	***	ns

Data are mean ± SEM for n=8 rats *per* group; Significance level: not significant (ns), $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; means in the same row with different superscripts are significantly different ($P<0.05$). Experimental diets: P = palm oil; PCLA = palm oil + 1% CLA; O = ovine fat; OCLA = ovine fat + 1% CLA. † these fatty acids co-elute.

5.3.2. Muscle fatty acid composition

Total FAME content in muscle was significantly lower in CLA supplemented animals (Table 5.4). The main fatty acids in the muscle, from all dietary groups, were 16:0, 16:1*c*9, 18:1*c*9, and 18:2*n*-6. All fatty acids were affected by fat source, except 20:2*n*-6 and 20:4*n*-6. Once more, these differences are firstly explained by the fatty acid composition of diets (Table 5.1). The total SFA content in muscle lipids was not affected by CLA factor, which can be due to the absence of effects on the two major saturated fatty acids, 16:0 and 18:0. CLA decreased MUFA sum. Palm oil groups presented more MUFA content in the muscle than ovine fat

groups. This trend was observed for 18:1*c*9, the main MUFA fatty acid. PUFA levels were diminished by CLA. CLA supplementation increased the total CLA content in 1.7 and 1.8 g/100 g fatty acids in vegetable and animal saturated fat diets, respectively. The *n*-3 PUFA sum was increased in ovine fat groups and no CLA effect was detected. CLA decreased *n*-6 PUFA sum, in particular, 18:2*n*-6, 20:3*n*-6, and 22:4*n*-6 fatty acids. The 18:3*n*-6 and 20:4*n*-6 were not affected by CLA supplementation in the diet. The sum of TFA increased with CLA supplementation. CLA affected majority of fatty acid levels, except for 16:0, 16:1*c*9, 18:0, 18:3*n*-6, 18:3*n*-3, 20:4*n*-6, 22:5*n*-3 and 22:6*n*-3. The Δ9-indexes 16 and 18 were not affected by CLA neither CLA × fat effects. Palm oil groups presented higher values of Δ9-index16, but lower values of Δ9-index18, comparing to ovine fat groups. Some fatty acids presented significant effects of CLA × fat interaction. CLA did not change 17:1*c*9 and 22:5*n*-6 in palm oil diets but increased its levels in ovine fat groups. On the contrary, 20:2*n*-6 was decreased in PCLA group. In both fat sources, 15:0 content increased but this change was greater in ovine fat. CLA diminished the 22:4*n*-6 content in a greater extent in palm oil diet.

Table 5.5 shows the composition of oleic and linoleic isomers in the muscle. The results showed that 18:1*t*11 and *c*11 were the major oleic isomers in muscle lipids. The fatty acids 18:1*t*9, *t*12 and *c*11 were lower in CLA supplemented groups than in P and O groups. The 18:1*t*10, *c*12 and *c*15 were increased by CLA only in palm oil diets. CLA increased the 18:2*t*8,*c*13/*c*9,*t*13 but decreased 18:2*t*9,*c*12. The 18:2*t*8,*c*12/*c*9,*t*12, *c*9,*c*15 and *c*12,*c*15 were not changed. The CLA isomeric profile in the muscle reflected, basically, the proportions of the various CLA isomers in the diets. CLA supplementation promoted an increase in most of the isomers content, excluding 18:2*t*12,*t*14, *t*11,*t*13, *c*/*t*12,14, *t*11,*c*13, *c*11,*t*13 and *t*7,*c*9 isomers. Similarly to the liver, the most prevalent isomer in all groups was the *c*9,*t*11 CLA isomer. In contrast, the percentage of *t*10,*c*12 CLA isomer was much lower, but, nevertheless, the second most prevalent isomer found in diets supplemented with CLA.

Table 5.4 Effects of CLA supplementation and fat source on total fatty acid content (g/100 g muscle) and composition (g/100 g total FAME) in the muscle of obese Zucker rats.

						Significance level		
	P	**PCLA**	**O**	**OCLA**	**SEM**	**CLA**	**Fat**	**CLA × Fat**
Total FAME	10.7	8.80	9.26	7.29	0.639	**	*	ns
14:0	1.20	1.34	1.36	1.48	0.040	**	***	ns
15:0	0.10^{c}	0.11^{bc}	0.21^{ab}	0.24^{a}	0.003	***	***	**
16:0	24.2	24.2	19.2	19.0	0.253	ns	***	ns
16:1*c*7	0.42	0.57	0.47	0.64	0.010	***	***	ns
16:1*c*9	9.57	9.77	8.18	7.77	0.173	ns	***	ns
17:1*c*9	0.12^{c}	0.12^{c}	0.39^{b}	0.42^{a}	0.005	**	***	**
18:0	2.52	2.28	3.95	3.95	0.087	ns	***	ns
18:1*c*9	36.4	35.3	33.6	32.8	0.324	**	***	ns
Σ i-18:1	5.88^{b}	6.08^{b}	9.23^{a}	9.02^{a}	0.123	ns	***	*
18:2*n*-6	13.6	12.6	12.4	11.7	0.153	***	***	ns
Σ nc-18:2	0.46^{c}	0.50^{c}	1.56^{a}	1.48^{b}	0.015	ns	***	**
Σ CLA	0.08	1.75	2.52	4.33	0.034	***	***	*
18:3*n*-3	0.04	0.03	0.04	0.04	0.001	***	***	ns
18:3*n*-6	0.05	0.05	0.04	0.04	0.002	ns	***	ns
20:0	0.20	0.14	0.17	0.12	0.004	***	***	ns
20:2*n*-6	0.13^{a}	0.11^{b}	0.12^{b}	0.11^{b}	0.003	***	ns	**
20:3*n*-6	0.34	0.25	0.27	0.23	0.012	***	***	ns
20:4*n*-6	2.30	2.14	2.07	2.02	0.136	ns	ns	ns
20:5*n*-3	0.03	0.02	0.04	0.03	0.002	*	***	ns
22:4*n*-6	0.33^{a}	0.22^{b}	0.18^{c}	0.13^{d}	0.011	***	***	*
22:5*n*-3	0.31	0.26	0.13	0.11	0.015	*	***	ns
22:5*n*-6	0.12^{b}	0.13^{b}	0.29^{a}	0.38^{a}	0.016	**	***	*
22:6*n*-3	0.30	0.33	0.57	0.62	0.030	ns	***	ns
Σ unidentified	1.43	1.63	2.61	2.94	0.042	***	***	ns
Σ SFA	28.2	28.1	24.9	24.8	0.300	ns	***	ns
Σ MUFA	52.4	52.1	51.9	50.6	0.287	***	***	ns
Σ PUFA	18.1	18.4	20.2	21.2	0.253	***	ns	ns
Σ *n*-3	0.68	0.64	0.78	0.80	0.046	ns	***	ns
Σ *n*-6	16.0	15.7	15.2	14.3	0.219	***	***	ns
Σ TFA	0.44	0.49	0.89	1.03	0.046	*	***	**
Δ9-index16	0.72	0.71	0.70	0.71	0.004	ns	*	ns
Δ9-index18	0.06	0.06	0.11	0.11	0.003	ns	***	ns

Data are mean ± standard error of mean (SEM) for n=8 rats *per* group; Significance level: not significant (ns), $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; means in the same row with different superscripts are significantly different ($P<0.05$). Experimental diets: P = palm oil; PCLA = palm oil + 1% CLA; O = ovine fat; OCLA = ovine fat + 1% CLA; nc, non-conjugated; CLA, conjugated linoleic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, *trans* fatty acids.

Σ i-18:1=18:1*t*6/*t*7/*t*8, *t*9, *t*10, *t*11, *t*12, *c*11, *c*12, *c*13, *t*6/*c*14, *c*15; Σ nc-18:2=18:2*t*9,*t*12, *t*8,*c*12/*c*9,*t*12, *t*8,*c*13/*c*9,*t*13, *t*9,*c*12, *t*11,*c*15, *c*9,*c*15, *c*12,*c*15; Σ CLA=*t*12,*t*14, *t*11,*t*13, *t*10,*t*12, *t*9,*t*11, *t*8,*t*10, *t*7,*t*9, *c*/*t*12,14, *t*11,*c*13, *c*11,*t*13, *t*10,*c*12, *c*9,*t*11, *t*8,*c*10, *t*7,*c*9; Σ SFA=14:0, 15:0, 16:0, 18:0, 20:0; Σ MUFA= 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, Σ i-18:1; Σ PUFA= 18:2*n*-6, Σ nc-18:2, Σ CLA, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-6, 22:5*n*-3, 22:6*n*-3; Σ *n*-3= 18:3*n*-3, 20:5*n*-3, 22:5*n*-3, 22:6*n*-3; Σ *n*-6= 18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 22:4*n*-6, 22:5*n*-6; Σ TFA= 18:1*t*6/*t*7/*t*8, *t*9, *t*10, *t*11, *t*12, *t*6/*c*14, 18:2*t*9,*t*12, *t*8,*c*12/*c*9,*t*12, *t*8,*c*13/*c*9,*t*13, *t*9,*c*12; Δ9-index16 = 16:0/(16:0 + 16:1*c*9); Δ9-index18 = 18:0/(18:0 + 18:1*c*9).

Table 5.5 Effects of CLA supplementation and fat source on oleic and linoleic isomers (g/100 g total FAME) in the muscle of obese Zucker rats.

						Significance level		
	P	**PCLA**	**O**	**OCLA**	**SEM**	**CLA**	**Fat**	**CLA × Fat**
18:1 isomers								
*t*6/*t*7/*t*8†	0.02	0.03	0.17	0.16	0.005	ns	***	ns
*t*9	0.09	0.08	0.31	0.29	0.006	*	***	ns
*t*10	0.05^{c}	0.22^{b}	0.44^{a}	0.44^{a}	0.020	***	***	***
*t*11	0.04	0.06	2.08	2.10	0.023	ns	***	ns
*t*12	0.07	0.08	0.31	0.36	0.010	**	***	ns
*c*11	5.39	5.05	4.56	4.34	0.058	***	***	ns
*c*12	n.d.	0.30^{b}	0.84^{a}	0.85^{a}	0.027	***	***	***
*c*13	0.16	0.15	0.24	0.24	0.009	ns	***	ns
*t*16/*c*14†	0.05^{d}	0.07^{c}	0.21^{a}	0.17^{b}	0.005	ns	***	***
*c*15	0.01^{c}	0.04^{b}	0.07^{a}	0.07^{a}	0.004	*	***	***
18:2 *n*-6 isomers								
non-conjugated								
*t*9,*t*12	n.d.	0.02^{b}	0.04^{a}	0.04^{a}	0.002	***	***	***
*t*8,*c*12/*c*9,*t*12†	n.d.	n.d.	0.40	0.39	0.004	ns	***	ns
*t*8,*c*13/*c*9,*t*13†	0.18	0.21	0.26	0.28	0.005	***	***	ns
*t*9,*c*12	0.28	0.27	0.12	0.10	0.004	**	***	ns
*t*11,*c*15	n.d.	n.d.	0.60^{a}	0.54^{b}	0.006	***	***	***
*c*9,*c*15	n.d.	n.d.	0.09	0.08	0.004	ns	***	ns
*c*12,*c*15	n.d.	n.d.	0.05	0.05	0.001	ns	***	ns
conjugated								
*t*12,*t*14	n.d.	n.d.	0.007^{a}	0.006^{b}	<0.001	*	***	*
*t*11,*t*13	n.d.	n.d.	0.024^{a}	0.018^{b}	<0.001	***	***	***
*t*10,*t*12	0.004	0.061	0.010	0.060	0.002	***	ns	ns
*t*9,*t*11	0.009	0.052	0.076	0.107	0.004	***	***	ns
*t*8,*t*10	0.004^{b}	0.019^{a}	0.005^{b}	0.013^{a}	0.002	***	ns	*
*t*7,*t*9	0.002	0.011	0.014	0.020	0.002	***	***	ns
c/*t*12,14†	n.d.	n.d.	n.d.	n.d.	-	-	-	-
*t*11,*c*13	0.001	n.d.	0.112	0.102	0.006	ns	***	ns
*c*11,*t*13	n.d.	n.d.	n.d.	n.d.	-	-	-	-
*t*10,*c*12	0.004^{c}	0.365^{b}	n.d.	0.434^{a}	0.011	***	**	**
*c*9,*t*11	0.057	1.18	2.17	3.36	0.032	***	***	ns
*t*8,*c*10	n.d.	0.058^{b}	n.d.	0.142^{a}	0.002	***	***	***
*t*7,*c*9	n.d.	n.d.	0.102^{a}	0.069^{b}	0.007	*	***	*

Data are mean ± standard error of mean (SEM) for n=8 rats *per* group; Significance level: not significant (ns), $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; means in the same row with different superscripts are significantly different ($P<0.05$). Experimental diets: P=palm oil; PCLA= palm oil + 1% CLA; O=ovine fat; OCLA= ovine fat + 1% CLA. † these fatty acids co-elute.

5.4. DISCUSSION

By using diets enriched in palm oil or ovine fat in our animal feeding experiment, it was possible to exploit the effect of CLA on the deposition of different fatty acids that are commonly present in human diet. The resulting fatty acid profiles were significantly modified by fat source. Ovine fat contrast palm oil because it contains a large number of fatty acids derived from rumen metabolism that include *trans* and *cis* isomers of oleic acid and conjugated and non-conjugated *trans* isomers of linoleic acid. Additionally, the ovine fat used

in this study came from animals reared with forage enriched in seed oils. As a result, the diets presented a more complete fatty acid profile richer in TFA, PUFA and CLA isomers than those from animals fed conventional diets (Bessa *et al.*, 2005, 2007). Fatty acid profiles in liver and muscle indicate that these isomeric fatty acids are extensively incorporated in tissues.

The fatty acids compositions of liver and muscle were modulated by the dietary intake of CLA. The liver levels of 16:0 and 18:0 increased in CLA supplemented groups. However, their metabolites, 16:1*c*9 and 18:1*c*9, remained constant, except the 16:1*c*9 that was inferior in OCLA group comparing with O group. On the contrary, in muscle, 16:0, 18:0 and 16:1*c*9 were not affected but 18:1*c*9 levels were diminished by CLA. In the present study, the Δ9-indexes were calculated in order to predict the SCD activity in liver and muscle. One of the mechanism which CLA may alter the lipid metabolism is the inhibition of SCD activity or mRNA expression in tissues (Park *et al*., 2000; Lin *et al*., 2004; Viswanadha *et al*., 2006). The SCD enzyme is the rate-limiting enzyme in the biosynthesis of MUFA. Preferentially, it catalyses the conversion of 16:0 and 18:0 to 16:1*c*9 and 18:1*c*9, respectively, by introduction of a *cis* double bond at the Δ9 position (Ntambi, 1999). The results suggested that CLA was particularly effective inhibiting SCD activity in liver when supplemented to the ovine fat diet. In muscle, the absence of CLA effect on these ratios may be a result from a more sensibility of the liver, the main organ of lipid metabolism (Chow, 2000).

Linoleic and α-linolenic (18:3*n*-3) acids are essential fatty acids metabolised to arachidonic (20:4*n*-6) and docosahexaenoic (22:6*n*-3) acids by Δ6-desaturase, elongase and Δ5-desaturase enzymes. In the same manner, *c*9,*t*11 and *t*10,*c*12 CLA isomers compete for these enzymes and are metabolised to conjugated diene metabolites that have been shown to inhibit lipoprotein lipase activity and stimulate glycerol release in 3T3-L1 adipocytes, similarly to CLA (Park *et al.*, 2005). Additionally to alter desaturation activities, CLA may compete with other PUFA to be incorporated into the membranes phospholipids (Kramer *et al.*, 1998). Dietary CLA was incorporated in the liver at the expenses of linoleic acid (Belury & Kempa-Steczko, 1997; Kelley *et al*., 2004; Zabala *et al.*, 2006) and arachidonic acid (Bulgarella *et al*., 2001; Sanders *et al.*, 2004). In this work, sunflower oil replaced CLA oil in the diets without supplementation, and a reduction of 18:2*n*-6 in CLA supplemented groups was already expected. For all dietary groups, similar arachidonic acid levels were observed in the muscle. Surprising, CLA increased the percentage of this fatty acid in the liver. Low levels of α-linolenic acid were present in all groups from both tissues, except livers from OCLA group

that presented higher levels of this fatty acid comparing to O group. The *n*-3 PUFA 22:5*n*-3 and 22:6*n*-3, that are prevalent in membrane phospholipids, were higher in OCLA livers than in O livers. This may be a result from the fact that ovine fat possess more 18:3*n*-3. Additionally, as CLA diminished FAME content in the liver, it is expected that the proportion of triacylglycerols to phospholipids may also decrease.

The *trans* and *cis* isomers of oleic acid and conjugated and non-conjugated *trans* isomers of linoleic acid, are typical of ruminant tissues. Therefore, some of these fatty acids only appeared in liver and muscle from ovine fat groups. Ovine fat diets promoted an incorporation of these minor isomers whose biological effects are not completely understood. In human diets, the most common TFA belongs to the group of *trans* 18:1, being the vaccenic acid (18:1*t*11) the most representative (Kraft *et al.*, 2006). TFA intake has been associated with cardiovascular diseases, type 2 diabetes and various cancer types, and it is recommended to contribute less than 1% of total energy intake (WHO, 2003). However, due to the bioconversion of vaccenic acid into *c*9,*t*11 CLA isomer by Δ9-desaturase, 18:1*t*11 should be consider a neutral or beneficial *trans* isomer (Prates & Bessa, 2009). Butter naturally enriched in 18:1*t*11 and supplemented with *c*9,*t*11 CLA isomer reduced lipid deposition in rabbits aorta (Roy *et al.*, 2007). In addition, 18:1*t*11 modified the serum lipid profile of growing pigs by increasing some fatty acids levels associated with protector effects of coronary heart diseases (Haug *et al.*, 2008). In the muscle, CLA did not change 18:1*t*11 contents for both fat sources, but in the liver CLA increased its levels when supplemented to the ovine fat diet. This enhancement may be due to the higher 18:1*t*11 content in OCLA diet than in O diet. Furthermore, OCLA diet provided more CLA than any other diet tested and the eventual inhibition of SCD enzyme could have promoted higher 18:1*t*11 content. It was also observed an unexpected increase of 18:1*t*10 content in the liver with CLA supplementation. In the muscle, this increase was limited to PCLA diet.

The CLA isomeric profiles of both tissues, in part reflect the CLA isomeric profile present in the diets. However, different depositions of some isomers was observed. Interestingly, there was a preferential accumulation of 18:2*t*11,*t*13 and *c*/*t*11,13 isomers in muscle comparing to liver. Moreover, Kramer *et al*. (1998) reported a higher content of 18:2*c*11,*t*13 in heart lipids than in liver. Also, the levels of *t*10,*c*12 CLA isomer were higher in muscle than in liver. Despite equal amounts of *c*9,*t*11 and *t*10,*c*12 CLA isomers in CLA supplement, its respective proportions found in liver and muscle were quite different. In both tissues, *c*9,*t*11 was more efficiently incorporated than *t*10,*c*12, as reported by other investigators (Kelley *et al.*, 2006;

Martin *et al.*, 2007). Li *et al.* (1998) found more *c*9,*t*11 than *t*10,*c*12 in liver, serum, bone and marrow. On the contrary, spleen, muscle and heart contained more *t*10,*c*12 than *c*9,*t*11 CLA isomer. Recently, Tsuzuki and Ikeda (2007) observed a similar extent of lymphatic recovery of these two isomers in rats, suggesting that geometrical and positional isomerism of the conjugated double bounds had no influence on the absorption by the small intestine. Previous research has shown that CLA can be further desaturated and elongated (Sébédio *et al.*, 2001) and some of the CLA ingested is likely oxidised for energy production. The *t*10,*c*12 CLA isomer has been reported to activate the β-oxidation system more strongly (Pariza *et al.*, 2001; Evans *et al.*, 2002) and to be more susceptible to oxidation (Tsuzuki *et al.*, 2004) than c9,t11 CLA isomer.

5.5. CONCLUSIONS

In a general overview of this study, the fatty acid profile on liver and muscle of rats fed palm oil and ruminant fat were found to contain interesting interaction between CLA and fat source. The modification of Δ9-desaturase index showed a different influence on Δ9-desaturase activity in liver and muscle by CLA. Through unclear molecular pathways, CLA also increased the 18:1*t*10 content. There were significant changes in *n*-6 and *n*-3 hepatic and muscle fatty acids when CLA were fed to animals. The percentage of *c*9,*t*11 CLA isomer was higher in liver than in muscle. On the contrary, *t*10,*c*12 CLA isomer was twofold high in muscle that in liver, which reveals different metabolisation pathways of CLA isomers in both tissues. These results have shown that CLA supplementation in obese Zucker rats altered the fatty acid profile and was dependent upon dietary fat source.

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CHAPTER 6 Serum adipokine profile and fatty acid composition of adipose tissue are affected by conjugated linoleic acid and saturated fat diets in obese Zucker rats

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Abstract

Conjugated linoleic acid (CLA) has been reported as having body fat lowering properties and the ability to modulate the inflammatory system in several models. In the present study, the effects of CLA added to saturated fat diets, from vegetable and animal origins, on the serum adipokine profile of obese Zucker rats were assessed. In addition, the fatty acid composition of epididymal and retroperitoneal fat depots was determined and a principal component analysis (PCA) was used to assess possible relationships between fatty acids and serum metabolites. Atherogenic diets (2% cholesterol) were formulated with palm oil and ovine fat and supplemented or not with 1% of a mixture (1:1) of *c*9,*t*11 and *t*10,*c*12 CLA isomers. CLA fed animals exhibited lower daily feed intake, final body and liver weights, and hepatic lipids content. Total and LDL-cholesterol levels were increased in CLA supplemented groups. CLA also promoted higher adiponectin and lower PAI-1 serum concentrations. In contrast to palm oil diets, ovine fat increased insulin resistance and serum levels of leptin, TNF-α and IL-1β. Epididymal and retroperitoneal fat depots had similar deposition of individual fatty acids. The PCA analysis showed that the *t*10,*c*12 CLA isomer was highly associated with adiponectin and PAI-1 levels. Summing up, CLA added to vegetable saturated enriched diets, relative to those from animal origin, seems to improve the serum profile of adipokines and inflammatory markers in obese Zucker rats due to a more favourable fatty acid composition.

6.1. INTRODUCTION

The metabolic disorders caused by overweight are one of the major factors contributing to the increase of health-care costs. Among these disorders are atherosclerosis, hypertension, insulin resistance, hypertriglyceridaemia and hypercholesterolaemia (Murdolo & Smith, 2006). Dysregulated endocrine function of adipose tissue, in particular of visceral compartment, leads to an increased release of hormones and pro/anti-inflammatory molecules (Murdolo & Smith, 2006). These factors secreted by adipose tissue, although not exclusively by adipose cells (Fain, 2006), are called adipokines. They include adiponectin, leptin, tumour necrosis factor-α (TNF-α), interleukins-1β (IL-1β) and -6 (IL-6), C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1), being most of them elevated in direct proportion to adiposity, except adiponectin (Wang *et al.*, 2008). Adiponectin, leptin and ghrelin are involved in energy balance, regulating feeding behaviour (Vendrell *et al.*, 2004). Despite ghrelin being mainly produced in the stomach, visceral adipose tissue also secretes this hormone (Dolinková *et al.*, 2008). Enhanced activities of TNF-α and IL-6 are involved in the development of obesity-related insulin resistance, and PAI-1 in the impairment of fibrinolysis (Trayhurn, 2005). Other adipokines, like adiponectin and leptin, mitigate insulin resistance as they stimulate β-oxidation of fatty acids in skeletal muscle (Vendrell *et al.*, 2004). The understanding of adipokines molecular actions may lead to effective therapeutic strategies, ultimately designed to protect obese individuals against cardiovascular diseases, hypertension and type 2 diabetes.

Conjugated linoleic acid (CLA) is a group of positional and geometric isomers of linoleic acid with conjugated double bonds, that modulates adiposity and related adipokine levels (Yamasaki *et al.*, 2003). CLA is naturally found in foods of ruminant origin (*e.g.* beef, lamb, and dairy products), and is composed mainly by *c*9,*t*11, with *t*10,*c*12 CLA isomer comprising only a small percentage (Martins *et al.*, 2007). It is well established that CLA has important effects on glucose and lipid metabolisms and that, especially the *t*10,*c*12 CLA isomer, is able to reduce fat deposition (Wang *et al.*, 2006b; Park *et al.*, 2007a). However, it has been reported that CLA, as a mixture or its *t*10,*c*12 CLA isomer, induces potential side effects, such as, hyperinsulinaemia and insulin resistance, promoting hepatic steatosis in lean and obese mouse models (Wendel *et al.*, 2008; Halade *et al.*, 2009). Additionally to CLA's adipokine modulation, other dietary fatty acids may interfere with adipokines expression and concentration (Ukropec *et al.*, 2003; Kim *et al.*, 2009). Nevertheless, the relationship between

CLA, adipokines and fatty acids in adipose tissue is far from clear in obese insulin-resistant models. Mutations affecting leptin action are associated with massive obesity in both human subjects and rodents (Montague *et al.*, 1997). For this purpose, the present study selected the *fa*/*fa* Zucker rat as experimental model, which develops morbid obesity due to the *fa* mutation on the leptin receptor. These rats display mild hyperglycaemia, pronounced hyperinsulinaemia, marked reduction in insulin sensitivity and hepatic steatosis (Kasiske *et al.*, 1992). Dietary manipulations, involving protein type and fat levels, result in complicated interactions with the fat-reducing effect of CLA (Akahoshi *et al.*, 2005). This approach might help to support the beneficial effects of CLA and minimise its possible unfavourable side effects. Western diets provide a dramatic dietary fat imbalance, characterised by high percentages of saturated fats, which are among the most important causes of human mortality in developed countries (Hu *et al.*, 2001). To the best of our knowledge, the combination of dietary saturated fats from animal and vegetable origins with CLA effects has not been evaluated so far. Facing this scenario, the main goal of this study was to search for CLA and saturated fat diets effects on the serum adipokine profile of obese Zucker rats. High fat diets were formulated as atherogenic (with 2% of cholesterol) based on two distinct saturated fats (palm oil or ovine fat) alone or combined with 1% of CLA. Moreover, a principal component analysis was used in order to elucidate possible associations between the levels of individual fatty acids from adipose tissue and serum metabolites.

6.2. EXPERIMENTAL PROCEDURES

6.2.1. Diet ingredients

All dietary components, except CLA oil and ovine fat, were purchased from Provimi Kliba SA (Kaiseraugst, Switzerland), which prepared and pelleted the experimental diets. The CLA oil (80% purity) was a generous gift of PharmaNutrients Inc. (Gurnee, IL, USA) and contained a 1:1 mixture of *c*9,*t*11 and *t*10,*c*12 CLA isomers. The ovine peritoneal fat was obtained from lambs fed with pelleted dehydrated lucerne supplemented with 6% of a blend of sunflower and linseed oils (Jerónimo *et al.*, 2009). The raw ovine fat was melted, and then filtered to subsequent incorporation in the diets.

6.2.2. Animals and diets

Experimental procedures were reviewed by the Ethics Commission of CIISA/FMV and approved by the Animal Care Committee of the National Veterinary Authority (Direcção-Geral de Veterinária, Portugal), following the appropriated European Union guidelines (N. 86/609/EEC). Obese male Zucker rats (*n*=32, Harlan Interfauna Iberia, Barcelona, Spain), ageing 5 weeks old, were individually housed in cages and maintained on a 12 h light-dark cycle at 22 ± 2 ºC. All rats had free access to tap water and semi-purified atherogenic diets, based on the AIN-93G formulation. Ingredients composition (% feed) was casein (20.0), dextrose (13.2), sucrose (11.9), corn starch (29.3), cellulose (5.0), AIN-93G vitamin-mixture (0.5), AIN-93G mineral-mixture (2.4), amino acids (0.3), cholesterol (2.0), cholic acide sodium salt (0.5) and BHT (0.01). After an acclimatisation period of 1 week, eight rats were allocated to one of the following dietary treatments: group P - 11.3% of palm oil plus 3.8% sunflower oil; group PCLA - 11.3% of palm oil plus 2.5% sunflower oil plus 1.2% CLA; group O - 11.3% ovine fat plus 3.8% sunflower oil; group OCLA - 11.3% ovine fat plus 2.5% sunflower oil plus 1.2% CLA. The composition of the diets are shown in Table 6.1 and the detailed fatty acid profile in Table 5.1 (page 68). Body weight and feed intake were recorded twice a week. After 14 weeks, rats were fasted for 12 h and killed by decapitation, under light isofluorane anaesthesia. The trunk blood was centrifuged (1500 g for 10 min, at room temperature) to separate serum. Following blood collection, the organs were removed, weighted and stored at -80 ºC.

6.2.3. Serum biochemical assays

Total cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerols, glucose, total proteins, aspartate aminotransferase (AST, EC 2.6.1.1), alanine aminotransferase (ALT, EC 2.6.1.2) and alkaline phosphatase (ALP, EC 3.1.3.1) were analysed in serum through diagnostic test kits (Roche Diagnostics, Mannheim, Germany), using a Modular Hitachi Analytical System (Roche Diagnostics). VLDL-cholesterol and total lipids were calculated, according to Friedewald *et al.* (1972) and Covaci *et al.* (2006) formulas, respectively. Insulin, leptin, IL-1β, IL-6, TNF-α, MCP-1 and total PAI-1 concentrations in serum were determined simultaneously through the Rat Serum Adipokine LINCOplex kit (RADPK-81K, Linco Research, Millipore, MA, USA), using the Luminex xMAP technology (Lincoplex 200, Linco Research). Adiponectin (EZRADP-62K, Linco Research), ghrelin (EZRGRT-91K, Linco

Research) and CRP (CYT 294, Chemicon International, Millipore) levels were measured using commercial ELISA kits. The degree of insulin resistance was calculated by the homeostasis model assessment using the insulin resistance index (HOMA-IR) (Matthews *et al.*, 1985): fasting serum glucose (mmol/l) times fasting serum insulin (mU/l) divided by 22.5. Low HOMA-IR values indicate high insulin sensitivity, whereas high HOMA-IR values indicate high insulin resistance.

Table 6.1 Composition of the experimental diets.

	P	**PCLA**	**O**	**OCLA**
Ingredients (% feed)				
Casein	20.0	20.0	20.0	20.0
Dextrose	13.2	13.2	13.2	13.2
Sucrose	11.9	11.9	11.9	11.9
Corn starch	29.3	29.3	29.3	29.3
Cellulose	5.0	5.0	5.0	5.0
Vitamin-mixture	0.5	0.5	0.5	0.5
Mineral-mixture	2.4	2.4	2.4	2.4
Amino acids	0.3	0.3	0.3	0.3
Palm oil	11.3	11.3	–	–
Ovine fat	–	–	11.3	11.3
Sunflower oil	3.8	2.5	3.8	2.5
CLA	–	1.2	–	1.2
Cholesterol	2.0	2.0	2.0	2.0
Cholic acide sodium salt	0.5	0.5	0.5	0.5
BHT	0.01	0.01	0.01	0.01
Proximate composition (% dry matter)				
Crude protein	18.0	18.0	18.0	18.0
Crude fat	15.0	15.0	15.0	15.0
Crude ash	3.5	3.5	3.5	3.5
Crude fibre	3.5	3.5	3.5	3.5
Nitrogen-free extract	50.0	50.0	50.0	50.0

Dietary treatments: P = palm oil; PCLA = palm oil + 1% CLA; O = ovine fat; OCLA = ovine fat + 1% CLA.

6.2.4. Hepatic lipid extraction

After liver lyophilisation, total lipids were extracted, in duplicate, and gravimetrically measured using the procedure described by Fritsche *et al*. (2000). Briefly, lipids were extracted three times with methylene chloride/methanol (4:1 v/v) and a fourth time with *n*-hexane. Following evaporation and dry, the fatty residue was weighted.

6.2.5. Fatty acid composition of adipose depots

Fatty acids methyl esters (FAME) from epididymal and retroperitoneal adipose depots were obtained using the method of Christie *et al.* (2001), slightly modified by Raes *et al.* (2004). In short, 1 ml of dry toluene was added to 0.05 g of lyophilised samples and fatty acids were methylated through a base-catalysis followed by an acid-catalysis. At 50 ºC, sodium methoxide in anhydrous methanol (0.5 mol/l) reacted for 30 min, followed by hydrogen chloride in methanol (1/1 v/v), for 10 min. FAME were extracted twice with 3 ml of hexane and pooled extracts were evaporated until 2 ml volume, under a stream of nitrogen. The resulting FAME were then analysed by gas chromatography, using a fused-silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d. × 0.20 mm film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA), equipped with a flame-ionisation detector. The chromatographic conditions were described in detail by Jerónimo *et al.* (2009). Fatty acid composition was expressed as g/100 g of total fatty acids identified. The fatty acid composition of diets was obtained following the same analytical procedure described for adipose depots.

6.2.6. Statistics

Statistical analysis was carried out with the Statistical Analysis System (SAS) software package, version 9.1 (SAS Institute, Cary, NC, USA). All data were reported as mean with their standard errors of mean (SEM). The procGLM procedure was used to perform a 2 × 2 factorial analysis to determine significant main effects of CLA, fat source and their interaction (CLA × fat). When the interaction effect was significant, differences between groups were calculated using Tukey´s *post-hoc* test at $P<0.05$. In order to evaluate differences between fatty acid composition from epididymal and retroperitoneal adipose depots, the procGLM procedure was used with tissue as a single factor. Afterwards, a principal component analysis (PCA) was performed, using procPRINCOMP of SAS, to assess relationships between fatty acids (average between fatty acids from epididymal and retroperitoneal adipose depots) and adipokines. After data normalisation, the analysis was based on the correlation matrix (consisting of 29 variables) and principal components (PC) were considered as significant if they contributed more than 5% for the total variance.

6.3. RESULTS

6.3.1. Body composition

The daily feed intake and body weight, as well as both fat depots and liver weights, are presented in Table 6.2. CLA fed rats had lower final body weight due, in part, to a decrease in the average daily intake induced by CLA ($P<0.01$). In the same manner, liver weight and hepatic lipids were reduced in PCLA and OCLA groups in relation to their matching groups ($P<0.001$). In contrast, neither CLA nor fat source had any effect on the weight of epididymal and retroperitoneal fat depots ($P>0.05$). The interaction CLA × fat was not observed for any of those parameters ($P>0.05$). Finally, the weights of *LD* muscle, kidney, testicle, spleen, heart and lung were did not presented statistical differences (data not shown).

6.3.2. Serum metabolite profile

The serum metabolite profile is also listed on Table 6.2. Animals fed CLA had higher levels of total and LDL-cholesterol ($P<0.05$) and lower glucose levels ($P<0.05$). Insulin and HOMA-IR were higher in ovine fat groups compared with palm oil groups ($P<0.05$), but were not affected by CLA ($P>0.05$). Regarding the hepatic enzymes, lower circulating AST concentrations were found for CLA supplemented animals ($P<0.05$). CLA enhanced adiponectin levels ($P<0.001$) regardless the fat source. Leptin levels were affected by the fat source but not by the supplementation with CLA, being higher in ovine fat groups ($P<0.001$). Neither CLA nor fat source had effects on ghrelin concentrations ($P>0.05$). The changes in the pro-inflammatory markers IL-1β, IL-6, TNF-α and CRP are also summarised on Table 6.2. IL-1β and TNF-α were affected by the fat source independently of CLA supplementation, being higher in ovine fat groups ($P<0.05$). IL-6 was dependent on the interaction between CLA and fat source. OCLA group presented higher values of IL-6 than the other dietary treatments ($P<0.05$). CRP and MCP-1 levels were similar for all dietary groups ($P>0.05$). In contrast, CLA reduced PAI-1 concentrations regardless the fat source ($P<0.01$). IL-1β and TNF-α were affected by the fat source independently of CLA supplementation, being higher in ovine fat groups ($P<0.05$). IL-6 was dependent on the interaction between CLA and fat source. OCLA group presented higher values of IL-6 than the other dietary treatments ($P<0.05$).

Table 6.2 Body composition parameters and serum metabolites.

						Significance level		
	P	**PCLA**	**O**	**OCLA**	**SEM**	**CLA**	**Fat**	**CLA × Fat**
Growth and body composition								
Initial body weight (g)	240	234	234	234	11.2	ns	ns	ns
Final body weight (g)	577	552	584	511	12.7	***	ns	ns
Daily feed intake (g/d)	23.7	23.0	23.9	21.8	0.508	**	ns	ns
Retroperitoneal fat weight (g)	24.0	24.7	23.7	20.7	1.62	ns	ns	ns
Epididymal fat weight (g)	13.7	15.2	14.7	14.6	0.605	ns	ns	ns
Liver weight (g)	62.2	54.7	60.6	48.1	1.88	***	*	ns
Hepatic lipids (% liver weight)	21.9	18.9	23.5	19.2	0.800	***	ns	ns
Biochemistry profile								
Total cholesterol (mg/dl)	1172	1517	1371	1627	133	*	ns	ns
HDL-cholesterol (mg/dl)	181	176	190	185	10.1	ns	ns	ns
LDL-cholesterol (mg/dl)	749	1000	921	1053	87.6	*	ns	ns
VLDL-cholesterol (mg/dl)†	88.5	121	87.0	106	16.9	ns	ns	ns
Triacylglycerols (mg/dl)	443	607	437	530	84.4	ns	ns	ns
Total lipids (mg/dl)‡	2049	2654	2356	2675	242	ns	ns	ns
Total proteins (g/dl)	7.87	7.17	7.58	7.02	0.326	ns	ns	ns
Glucose (mg/dl)	116	106	123	108	5.66	*	ns	ns
Insulin (ng/ml)	1.78	1.84	3.02	2.53	0.26	ns	**	ns
HOMA-IR (mmol/l×mU/l)§	14.1	14.4	25.5	18.4	2.68	ns	*	ns
Hepatic markers								
AST (U/l)	355	287	341	240	37.7	*	ns	ns
ALT (U/l)	69.4	85.9	78.0	88.9	6.93	ns	ns	ns
ALP (U/l)	284	283	226	237	33.2	ns	ns	ns
Serum adipokine profile								
Adiponectin (μg/ml)	11.6	15.3	10.9	14.6	0.716	***	ns	ns
Leptin (ng/ml)	3.61	3.03	6.22	5.48	0.688	ns	***	ns
Ghrelin (pg/ml)	1.22	1.06	0.75	1.06	0.184	ns	ns	ns
IL-1β (pg/ml)	175	196	451	448	49.4	ns	***	ns
IL-6 (pg/ml)	165^{b}	148^{b}	179^{b}	259^{a}	20.5	ns	**	*
TNF-α (pg/ml)	24.1	24.4	24.9	25.7	0.411	ns	*	ns
CRP (pg/ml)	250	320	286	292	33.7	ns	ns	ns
MCP-1 (pg/ml)	561	455	371	588	100	ns	ns	ns
PAI-1 (pg/ml)	370	243	560	202	77.0	**	ns	ns

Dietary treatments: P = palm oil; PCLA = palm oil + 1% CLA; O = ovine fat; OCLA = ovine fat + 1% CLA.
†VLDL-cholesterol = 1/5 [triacylglycerols]; ‡Total lipids = [total cholesterol] × 1.12 + [triacylglycerols] × 1.33 + 148. §HOMA-IR, insulin resistance index = [fasting serum glucose] × [fasting serum insulin] / 22.5. Statistical significance: ns = not significant, $P>0.05$; *$P<0.05$; **$P<0.01$; ***$P<0.001$; means in the same row with different superscripts are statistically different (Tukey´s *post-hoc*, $P<0.05$).

6.3.3. Fatty acid composition of epididymal and retroperitoneal adipose depots

The fatty acid composition of epididymal and retroperitoneal adipose depots are presented in Table 6.3 and Table 6.4, respectively. In both fat depots, the effect of fat source was detected for all fatty acids (P<0.001). Palm oil groups were richer in 16:0, 16:1*c*9, 18:1*c*9, 18:2*n*-6 and 20:4*n*-6 fatty acids than ovine fat groups (P<0.001). Rats fed ovine fat presented higher values of 16:1*c*7, 18:0, 18:1*t*10/*t*11, 18:3*n*-3 and CLA isomers than groups fed palm oil (P<0.001). CLA-fed rats had more 14:0, 16:1*c*7 and 18:1*t*10/*t*11, and less 18:1*c*9, 18:2*n*-6 and 20:4*n*-6 fatty acids (P<0.001). Moreover, CLA supplementation increased the percentages of *c*9,*t*11 CLA isomer (P<0.001), comparing with their matching groups in both fat depots. Also, *t*10,*c*12 and the other CLA isomers were increased in PCLA and OCLA groups (P<0.001) but in a lower magnitude.

Table 6.3 Fatty acid composition (% of total FAME) of epididymal adipose depot.

						Significance level		
	P	**PCLA**	**O**	**OCLA**	**SEM**	**CLA**	**Fat**	**CLA × Fat**
14:0	1.05	1.24	1.30	1.42	0.020	***	***	ns
16:0	25.2	25.69	18.80	19.00	0.167	ns	***	ns
16:1*c*7	0.42	0.51	0.52	0.66	0.013	***	***	ns
16:1*c*9	7.59^{a}	7.87^{a}	6.67^{b}	6.18^{c}	0.111	ns	***	**
18:0	1.98^{b}	1.77^{b}	4.19^{a}	4.27^{a}	0.065	ns	***	*
18:1*t*10/*t*11	n.d.	0.31	3.30	3.53	0.044	***	***	ns
18:1*c*9	40.9	39.4	36.5	35.3	0.198	***	***	ns
18:1*c*11	4.67^{b}	4.91^{a}	4.75^{b}	4.59^{b}	0.052	ns	**	***
18:2*n*-6	15.3	13.3	13.8	12.2	0.155	***	***	ns
18:3*n*-3	0.17	0.16	0.77	0.74	0.014	ns	***	ns
*c*9,*t*11 CLA	n.d.	1.55^{c}	2.37^{b}	4.09^{a}	0.038	***	***	*
*t*10,*c*12 CLA	n.d.	0.58	0.15	0.72	0.016	***	***	ns
CLA others	n.d.	0.03^{b}	n.d.	0.26^{a}	0.005	***	***	***
20:4*n*-6	0.67	0.31	0.56	0.25	0.017	***	***	ns
Σ FAME	98.1	97.6	93.7	93.3	0.079	***	***	ns
Σ Others	1.95	2.45	6.33	6.72	0.079	***	***	ns
Σ SFA	28.3	28.7	24.3	24.7	0.206	ns	***	ns
Σ MUFA	53.6	52.7	48.4	46.8	0.216	***	***	ns
Σ PUFA	16.2	13.7	15.2	13.2	0.162	***	***	ns
Σ CLA	n.d.	2.16^{c}	2.51^{b}	5.07^{a}	0.051	***	***	***
Δ9-index16	1.30^{b}	1.31^{b}	1.36^{a}	1.33^{b}	0.007	ns	***	*
Δ9-index18	21.8^{b}	23.4^{a}	9.74^{c}	9.28^{c}	0.356	ns	***	*

Dietary treatments: P = palm oil; PCLA = palm oil + 1% CLA; O = ovine fat; OCLA = ovine fat + 1% CLA. n.d. = not detected; FAME = fatty acid methyl esters. SFA, saturated fatty acids = sum of 14:0, 16:0 and 18:0; MUFA, monounsaturated fatty acids = sum of 16:1*c*7, 16:1*c*9, 18:1*c*9 and 18:1*c*11; PUFA, polyunsaturated fatty acids = sum of 18:2n-6, 18:3n-3 and 20:4n-6; Total CLA = sum of *c*9,*t*11, *t*10,*c*12 and CLA others; Δ9-index16, desaturation index 16:0 to 16:1 = 16:0/(16:0 + 16:1*c*9); Δ9-index18, desaturation index 18:0 to 18:1 = 18:0/(18:0 + 18:1*c*9). Statistical significance: ns = not significant, P>0.05; *P<0.05; **P<0.01; ***P<0.001; means in the same row with different superscripts are statistically different (Tukey´s post-hoc, P<0.05).

About fatty acids sums, CLA had no effect on total SFA (P>0.05) from epididymal fat depot but increased it in retroperitoneal fat depot (P<0.05). Total MUFA and total PUFA had lower percentages in CLA fed rats (P<0.001). In both depots, the Δ9-indices were affected by CLA × fat interaction (P<0.05). The Δ9-index16 ratio was higher in O group than the others (P<0.05), and P and PCLA groups had higher Δ9-index18 ratio than ovine fat groups (P<0.05). As shown in Table 6.3 and Table 6.4, the response on fatty acid composition of epididymal fat depot had a similar pattern as the one described for retroperitoneal fat depot. This fact was confirmed by the ANOVA with a single factor. Only 16:1*c*9 and 18:1*c*11 fatty acids had differences between fat depots (P<0.001, data not shown). Due to this similarity, the subsequent PCA statistical analysis was performed using the average values of fatty acids from epididymal and retroperitoneal fat depots.

Table 6.4 Fatty acid composition (% of total FAME) of retroperitoneal adipose depot.

						Significance level		
	P	**PCLA**	**O**	**OCLA**	**SEM**	**CLA**	**Fat**	**CLA × Fat**
14:0	1.07^{c}	1.27^{b}	1.40^{a}	1.48^{a}	0.024	***	***	*
16:0	25.1	25.8	18.1	18.4	0.180	**	***	ns
16:1*c*7	0.40	0.52	0.55	0.70	0.009	***	***	ns
16:1*c*9	6.81^{a}	6.80^{a}	6.09^{b}	5.37^{c}	0.114	**	***	**
18:0	1.95^{b}	1.72^{b}	4.35^{a}	4.38^{a}	0.061	ns	***	*
18:1*t*10/*t*11	n.d.	0.29	3.64	3.78	0.046	***	***	ns
18:1*c*9	40.0	39.1	35.8	35.0	0.129	***	***	ns
18:1*c*11	5.91	6.01	5.37	5.46	0.096	ns	***	ns
18:2*n*-6	15.6^{a}	13.1^{c}	13.9^{b}	12.2^{d}	0.134	***	***	**
18:3*n*-3	0.12	0.10	0.73	0.69	0.010	**	***	ns
*c*9,*t*11 CLA	0.05^{d}	1.70^{c}	2.51^{b}	4.39^{a}	0.032	***	***	**
*t*10,*c*12	n.d.	0.62	0.16	0.75	0.018	***	***	ns
CLA others	n.d.	0.05^{b}	n.d.	0.32^{a}	0.009	***	***	***
20:4*n*-6	0.73^{a}	0.29^{c}	0.62^{b}	0.27^{c}	0.017	***	***	*
Σ FAME	97.7	97.4	93.2	93.1	0.129	ns	***	ns
Σ Others	2.34	2.65	6.83	6.89	0.129	ns	***	ns
Σ SFA	28.1	28.8	23.8	24.2	0.211	*	***	ns
Σ MUFA	53.1	52.4	47.8	46.5	0.162	***	***	ns
Σ PUFA	16.4^{a}	13.5^{c}	15.3^{b}	13.2^{c}	0.138	***	***	**
Σ CLA	0.05^{d}	2.38^{c}	2.67^{b}	5.46^{a}	0.042	***	***	***
Δ9-index16	1.27^{bc}	1.26^{c}	1.34^{a}	1.29^{b}	0.006	***	***	**
Δ9-index18	21.5^{b}	23.7^{a}	9.25^{c}	8.98^{c}	0.330	**	***	***

Dietary treatments: P = palm oil; PCLA = palm oil + 1% CLA; O = ovine fat; OCLA = ovine fat + 1% CLA. n.d. = not detected; FAME = fatty acid methyl esters. SFA, saturated fatty acids = sum of 14:0, 16:0 and 18:0; MUFA, monounsaturated fatty acids = sum of 16:1*c*7, 16:1*c*9, 18:1*c*9 and 18:1*c*11; PUFA, polyunsaturated fatty acids = sum of 18:2*n*-6, 18:3*n*-3 and 20:4*n*-6; Total CLA = sum of *c*9,*t*11, *t*10,*c*12 and CLA others; Δ9-index16, desaturation index 16:0 to 16:1 = 16:0/(16:0 + 16:1*c*9); Δ9-index18, desaturation index 18:0 to 18:1 = 18:0/(18:0 + 18:1*c*9). Statistical significance: ns = not significant, P>0.05; *P<0.05; **P<0.01; ***P<0.001; means in the same row with different superscripts are statistically different (Tukey´s *post-hoc*, P<0.05).

6.3.4. Principal component analysis

The results of PC analysis of fatty acid composition of adipose depots and serum metabolites are shown in Table 6.5. The first five PC explained 87.56% of the total variance. The first and second PC were responsible for 63.48% of the total variance, 43.85 and 19.63%, respectively. The PC3 explained 10.08%, the PC4 7.64 % and the PC5 6.36% of the variability. As total variance explained by the first two PC is more than 50%, the projection of fatty acids and serum metabolites in the plane defined by these PC is shown in Figure 6.1.

Table 6.5 Loadings for the first five principal components (PC).

Variables	PC1	PC2	PC3	PC4	PC5
14:0	-0.83*	0.22	0.42	-0.09	-0.04
16:0	0.84*	0.49	-0.02	0.09	-0.11
16:1*c*7	-0.94*	0.23	0.02	-0.18	-0.09
16:1*c*9	0.88*	0.16	0.12	-0.27	-0.20
18:0	-0.91*	-0.36	0.00	0.06	0.13
18:1*t*10/*t*11	-0.94*	-0.29	0.08	-0.01	0.10
18:1*c*9	0.97*	0.12	-0.13	-0.01	-0.06
18:1*c*11	0.74	-0.04	0.12	-0.22	-0.29
18:2*n*-6	0.75	-0.56	-0.27	0.16	0.04
18:3*n*-3	-0.88*	-0.43	0.05	0.02	0.12
*c*9,*t*11 CLA	-0.97*	0.18	0.11	-0.09	0.01
*t*10,*c*12 CLA	-0.65	0.71*	0.13	-0.13	-0.09
CLA others	-0.77	0.40	-0.33	-0.10	0.00
20:4*n*-6	0.63	-0.68*	-0.21	0.17	0.04
Total cholesterol	-0.10	-0.59	-0.10	-0.63*	-0.24
HDL-cholesterol	-0.32	-0.01	-0.74*	0.14	-0.35
LDL-cholesterol	-0.15	-0.60	-0.18	-0.49	-0.42
Triacylglycerols	0.40	-0.05	0.44	-0.65*	0.45
Glucose	-0.01	-0.38	0.27	0.63*	-0.54
Insulin	-0.50*	-0.57*	0.27	-0.11	-0.37
Adiponectin	-0.23	0.71*	0.13	-0.13	-0.48*
Leptin	-0.75*	-0.61	0.17	0.04	0.08
Ghrelin	0.63*	0.02	-0.04	-0.36	0.27
IL-1β	-0.77*	-0.43	-0.03	0.18	0.17
IL-6	-0.31	-0.15	-0.68*	-0.53	-0.17
TNF-α	-0.67*	0.36	-0.52	0.04	0.03
CRP	-0.10	0.38	0.71*	-0.06	-0.39
MCP-1	-0.02	0.57	-0.43	0.02	-0.20
PAI-1	0.30	-0.76*	0.21	-0.04	-0.32
Portion of variance (%)	43.85	19.63	10.08	7.64	6.36
Cumulative variance (%)	43.85	63.48	73.56	81.2	87.56

* The most significant loadings.

Overall, PC1 was mainly characterised by 16:0 (0.84), 16:1*c*9 (0.88) and 18:1*c*9 (0.97) fatty acids on the right side and by 14:0 (-0.83), 16:1*c*7 (-0.94), 18:0 (-0.91), 18:1*t*10/*t*11 (-0.94), *c*9,*t*11 CLA (-0.97) and 18:3*n*-3 (-0.88) fatty acids on the left side. The fatty acid 16:0, mainly found in palm oil diets, is in opposition to 18:0 and 18:1 TFA mainly found in ovine fat diets (Table 5.1, page 68). Therefore, PC1 discriminated between fatty acids associated with palm oil and ovine fat added to the diets. The PC2 clearly distinguished *t*10,*c*12 CLA isomer (0.71) and adiponectin (0.71), located in the upper part, from PAI-1 (-0.76) and 20:4*n*-6 (-0.68), located in the lower part of the graphic. As the *c*9,*t*11 CLA isomer is already present in the ovine fat diets and the only source of *t*10,*c*12 CLA isomer was the supplementation, the PC2 discriminated between CLA supplementation and no CLA supplementation.

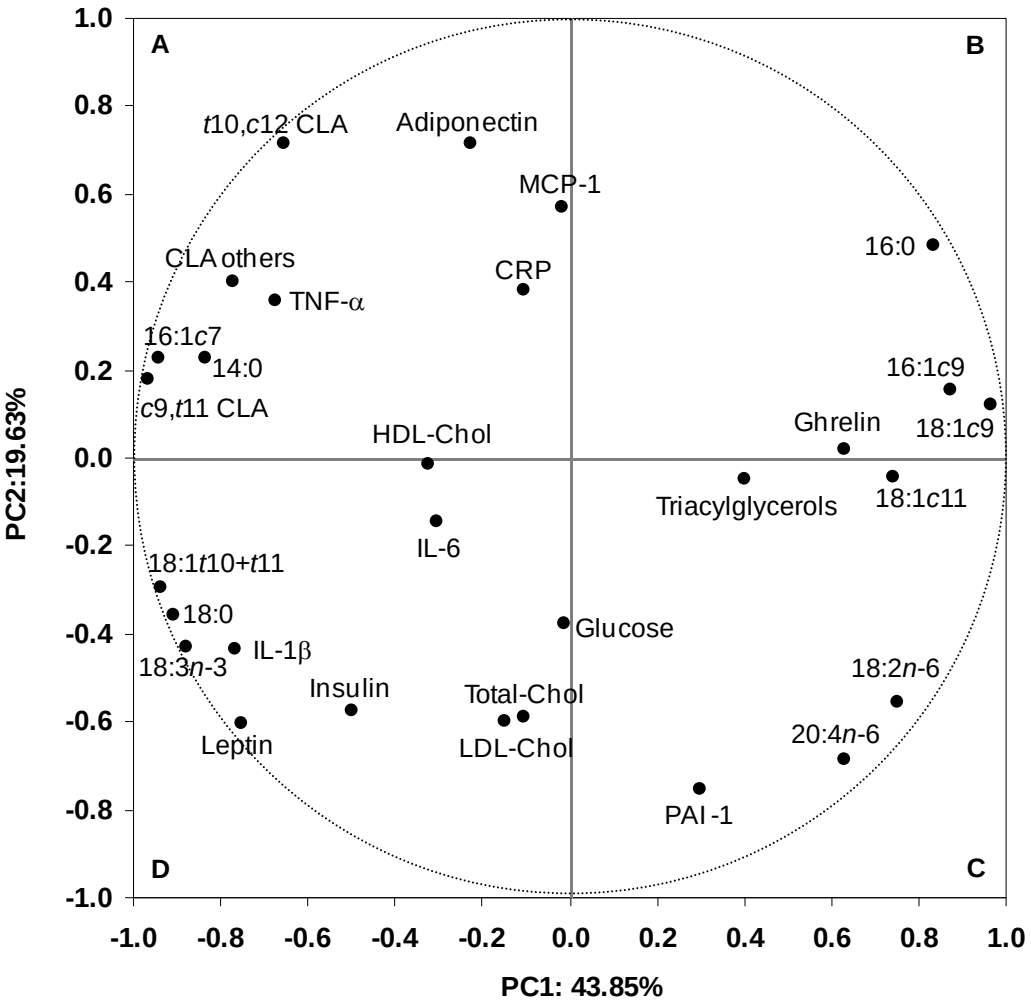

Figure 6.1 Projection of the variables (average of fatty acids from epididymal and retroperitoneal fat depots, and serum metabolites) in the plane defined by the two first principal components (PC1 and PC2 loadings plot).

In quadrant A, a group was defined by *c*9,*t*11 CLA isomer, other CLA isomers, 14:0, 16:1*c*7 and TNF-α. In quadrant B, another group was distinguished, composed by 16:1*c*9, 18:1*c*9,

18:1*c*11 and ghrelin. The fatty acids 18:0, 18:3*n*-3 and 18:1*t*10/*t*11, which showed enhanced values in ovine fat fed animals, appeared grouped in quadrant D and highly correlated with leptin, IL-1β and insulin. Interesting, this group of adipokines was opposed to 16:0; and the *t*10,*c*12 CLA isomer was opposed to 20:4*n*-6 and PAI-1; and the *c*9,*t*11 CLA isomer opposed to triacylglycerols. The variables with higher loadings were HDL-cholesterol (-0.74), IL-6 (-0.68) and CRP (0.71) in PC3; total cholesterol (-0.63), triacylglycerols (-0.65) and glucose (0.63) in PC4; and adiponectin (-0.48) in PC5.

6.4. DISCUSSION

The present investigation showed that animals fed CLA, regardless the fat source, gained less body weight, in parallel with a reduction in liver weight and hepatic lipids content. A decrease in daily feed intake by CLA was also observed, yet this variation seems insufficient to fully explain the variations in final body weight. Taking into account the conversion of food ingested on the weight gain, the average was 7.0 and 7.2 g food/g weight for palm oil and ovine fat groups. As the difference in feed intake was 68.6 and 205.8 g for P/PCLA and O/OCLA groups, respectively, only 9.8 and 28.6 g of the total weight gain is explained by the food, remaining to justify 15.2 and 44.4 g. In view of the fact that no other tissues weight were affected (though subcutaneous fat was not collected due to isolation difficulties), liver was likely the organ responsible for the less weight gain. It was also observed that the decrease in feed intake by CLA was not mediated by ghrelin. In agreement with our findings, other authors (Sisk *et al*., 2001; Nagao *et al*., 2005; Noto *et al*., 2006; Gudbrandsen *et al*., 2009) have reported a reduction of hepatic lipids content by CLA and similar fat depot weights.

Some authors reported CLA beneficial effects in obese Zucker rats by increasing plasma adiponectin levels and, therefore, alleviating hyperinsulinaemia (Nagao *et al.*, 2005; Noto *et al.*, 2007). Indeed, in the present study, CLA increased adiponectin and decreased glucose levels although without affecting insulin homeostasis. Instead, higher insulin and HOMA-IR values were found in rats fed ovine fat compared with rats fed palm oil. In this regard, the fat source effect in ovine fat diets, by rising up insulin levels, could overlap CLA reducing glucose effects. Also, independently of CLA supplementation, ovine fat diets increased the inflammatory markers TNF-α and IL-β although IL-6 only increased in the OCLA group. These inflammatory changes in ovine fat groups appeared insufficient to increase CRP and MCP-1 serum levels. In the present study, CLA had no effects on leptin levels, but ovine fat

based diets increased this adipokine. In contrast, Noto *et al*. (2007) and Halade *et al*. (2009) observed the ability of CLA to reduce leptin in serum. Alessi *et al*. (2003) reported that plasma levels of PAI-1 are closely related to the degree of liver steatosis than to the fat accumulation in adipose tissue. In the present work, PAI-1 showed lower levels in CLA supplemented groups, which possibly resulted from a decrease of hepatic lipids content. Serum levels of AST and ALT are normally increased in steatotic liver cases, and a stronger correlation between PAI-1 and AST rather than with ALT was reported by Alessi *et al*. (2003). In fact, our animals fed with CLA had a concomitant decrease of AST and PAI-1.

The administration of atherogenic diets allowed us to exploit if CLA is able to reverse the adverse affects of cholesterol, namely, in serum lipid profile and attenuating hepatic steatosis. Regarding the content of cholesterol in lipoproteins, CLA reduced VLDL and LDL fractions, as well as total cholesterol in obese Zucker rats (Noto *et al*., 2006). However, CLA induced the development of aortic fatty streaks in C57BL/6J mice (Munday *et al*., 1999). Surprisingly in the current study, CLA increased total and LDL-cholesterol, without affecting serum triacylglycerols. The changes found for cholesterol profile and PAI-1 led us to look for possible alterations in aorta microvasculature. The histological examination of different transversal segments of those aortas revealed only small morphological changes affecting smooth muscle cells, but no atheroma plaques were observed for any dietary group (images not shown).

The fatty acid composition of epididymal and retroperitoneal adipose depots showed similar patterns. CLA induced an increase in 16:1*c*7 and 18:1*t*10/*t*11 and a decrease in 18:1*c*9, 18:2*n*-6 and 20:4*n*-6 fatty acids. Sunflower oil (rich in 18:2*n*-6) replaced CLA oil in the diets without supplementation, and so, a reduction of 18:2*n*-6 in CLA supplemented groups was expected. This decrease probably also explain the reduiction in 20:4*n*-6 levels, an endogenous metabolite of 18:2*n*-6. The incorporation of CLA isomers into adipose tissue did not reflect the CLA profile of dietary treatments, being the *t*10,*c*12 CLA less incorporated than the *c*9,*t*11 CLA isomer, as observed by others (Kelley *et al.*, 2006). Apart from a possible endogenous synthesis in the rat, the *c*9,*t*11 CLA isomer levels in adipose tissue were mostly provided by the ovine fat-enriched diet. Park *et al*. (2000) reported a clear decrease of SCD enzyme activity by CLA. In both fat depots, it was not observed a clear effect of CLA on Δ9-indices (a proxy for SCD capability), but a CLA × fat interaction, meaning that CLA action depends on the fatty acid profile available in the diet.

In addition to the CLA ability to modulate adipokine metabolism affecting glucose and lipid metabolisms, namely, insulin sensitivity, other subgroups of fatty acids are capable of influencing these metabolisms, as well. In rats with hyperleptinaemia and hyperinsulinaemia, dietary *n*-3 PUFA caused a 40-50% reduction in plasma levels of leptin and insulin (Ukropec *et al.*, 2003). A recent work has demonstrated that 16:0 and 18:1*c*9 inhibited hepatic insulin signalling in hepatocyte cell cultures (Kim *et al.*, 2009). Nevertheless, there is insufficient knowledge about how fatty acid profiles of adipose tissue are correlated with serum metabolites concentration.

In the present study, a PCA was carried out to explore the relationships between the deposition of individual fatty acids in adipose tissue and serum metabolites. The projection of variables in the PC1 × PC2 plane (Figure 6.1), explaining a substancial percentage of the total variance (63%), revealed some interesting associations. Fatty acids distribution in the graph can be fairly interpreted by its common dietary origin, with quadrants A and D related with saturated fat source and quadrants A and B with CLA supplementation. Adiponectin was located close to *t*10,*c*12 CLA isomer and, on the opposite, PAI-1 which in turn was near 20:4*n*-6 and its precursor 18:2*n*-6. Indeed, a study in human subjects showed that dietary 18:2*n*-6 and 20:4*n*-6 were positively associated with PAI-1 activity (Byberg *et al.*, 2001). In relation to *c*9,*t*11 CLA isomer, this isomer appeared inversely related to triacylglycerols, which corroborates the reduction on plasma triacylglycerols levels in obese rodents fed this isomer (Ryder *et al.*, 2001; Roche *et al.*, 2002). TNF-α was located near the 14:0, 16:1*c*7 and *c*9,*t*11 CLA isomer in the lower region of quadrant A, reflecting the influence of both ovine fat and CLA supplementation. Leptin and insulin were located in left region of quadrant D, together with 18:0, 18:1*t*10/*t*11, 18:3*n*-3, suggesting that they are mainly related to fatty acids in ovine fat diets. Besides the fact that 16:0 has been associated with inflammation (Kennedy *et al.*, 2009; Petersson *et al.*, 2009), in the present study this predominant SFA in palm oil diets was located contrarily to IL-1β and IL-6. The location of ghrelin in the border of quadrants B and C showed that this hormone is mainly associated with dietary palm oil. The variables located near the origin of the PC1 and PC2 axis were not related with any of the studied factors. The associations between *t*10,*c*12 CLA, positively for adiponectin and negatively for PAI-1, reinforce our belief that *t*10,*c*12, but not *c*9,*t*11 CLA, is the main responsible for CLA effects observed in the present work. As it is not possible to draw definite causal relationships with these exploratory techniques of data analysis, some

associations should be exploited in well directed experimental designs. For instance, the effects of 18:2*n*-6 and 18:1*c*9 on serum ghrelin concentrations should be further evaluated.

In the present study, the specific effects observed for ovine fat treatments may be attributed to the different fatty acid composition of the diets containing fat from vegetable or animal sources (Table 5.1, page 68). In concrete, the influence on insulin resistance and pro-inflammatory profile can be mainly due to the imbalance between the pro-inflammatory 16:0 and 18:0 SFA (sum of 40-42 *vs*. 34%, for vegetable and animal fat diets, respectively) and the anti-inflammatory 18:1*c*9 MUFA (percentage of 32-35 *vs*. 21-22%, for vegetable and animal fat diets, respectively) present in the diet (Kennedy *et al*., 2009). Concerning *trans* fatty acids, 18:1*t*11 is the predominant in animal fats (~9% of total FAME), and is precursor of *c*9,*t*11 CLA isomer by endogenous conversion in tissues (Corl *et al*., 2003). In fact, it was recently reported that 18:1*t*11, in contrast to 18:1*t*10 isomer, is neutral or even beneficial to aortic lipid deposition in rabbits (Roy *et al*., 2007). Moreover, ovine fat diets contain minor amounts of *trans* fatty acids derived from rumen biohydrogenation, *trans* and *cis* isomers of oleic acid and conjugated and non-conjugated *trans* isomers of linoleic acid, not discriminated in the present study, which can display additional roles on those effects.

6.5. CONCLUSIONS

The results suggest that CLA supplementation of diets rich in saturated fats and cholesterol has some beneficial effects in obese Zucker rats by increasing adiponectin and decreasing PAI-1 serum levels, as well as alleviating hepatic steatosis through hepatic lipids reduction. However, this decrease does not seem to be enough to improve insulin sensitivity. The multivariate analysis suggested that CLA effects observed on adiponectin and PAI-1 levels can be mainly attributed to the *t*10,*c*12 CLA isomer. On the other hand, diets enriched in ovine fat appear to promote an increase in serum concentrations of some pro-inflammatory cytokines, also increasing insulin resistance. The present work questions the usefulness of CLA to prevent obesity in Western societies, where high intake of animal saturated fats is prevalent. Nonetheless, the combination of CLA, possibly *t*10,*c*12 CLA isomer, and saturated fat diets from vegetable origin seems to attenuate prejudicial effects promoted by animal fats.

Acknowledgments

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CHAPTER 7 Contrasting apoptotic responses of CLA in Zucker fatty rats fed palm oil and ovine fat

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Submitted

Abstract

In this study, we hypothesised that reducing weight properties of conjugated linoleic acid (CLA) are due to adipocyte apoptosis and that CLA may differentially modulate the apoptotic responses in liver lipotoxicity from rats fed saturated fat diets. Our experimental design included obese Zucker rats fed atherogenic diets with distinct fat origins, palm oil and ovine fat, and supplemented with CLA mixture. CLA induced no changes in adipose depots weight, in line with similar levels of apoptosis found in retroperitoneal fat. Yet, CLA reduced final body and liver weights. Interestingly, CLA had a contrasting effect on cell death in the liver according to the dietary fat. CLA increased hepatocyte apoptosis in rats fed palm oil diet compared with rats receiving palm oil diet alone. However, CLA reduced the apoptotic response in rats fed ovine fat, which in turn showed the highest levels of apoptosis in the liver. We next investigated the potential involvement of Fas death receptor, c-Jun N-terminal kinase (JNK) and endoplasmic reticulum (ER) signaling pathways. Liver apoptosis in rats receiving CLA and palm oil may occur through the extrinsic pathway without major contribution of JNK signaling. The ovine fat group displayed reduced production of ER-signaling proteins; nonetheless, CLA supplementation restored ER protein production and activated JNK, suggesting an adaptive response of survival that may explain the reduced levels of apoptosis. These findings reinforce the role of CLA as regulator of apoptosis in the liver. Moreover, the results highlight the importance of fatty acid composition of matrix-diets as a key factor in activation of apoptotic signaling pathways.

7.1. INTRODUCTION

Plenty of studies using laboratory animals, as well as cell culture systems have demonstrated the beneficial effects of conjugated linoleic acid (CLA) against atherosclerosis, hypertension, diabetes, inflammation, and some types of cancer (Prates & Bessa, 2009). Additionally, dietary CLA can affect weight control and interfere with body composition and adipocyte morphology (Lopes *et al.*, 2008), reducing white adipose mass, through distinct actions on fat deposition, lipolysis of adipose cells and lipid metabolism (Domeneghini *et al.*, 2006). The anti-obesity properties of CLA have been mostly attributed to a specific isomer, the *t*10,c12 CLA and partially ascribed to adipocytes apoptosis (Evans *et al.*, 2000; Hargrave *et al.*, 2000; Tsuboyama-Kasaoka *et al.*, 2000; Miner *et al.*, 2001; Hargrave *et al.*, 2004).

Apoptosis is an energy-dependent process of cellular deconstruction originally contrasted morphologically with necrosis. It represents a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells (Schwartzman & Cidlowski, 1993; Renehan *et al.*, 2001). Apoptosis might occur by several molecular pathways, which even if apparently independent, often cross-talk in many cell types. The best characterised and most prominent, however, are the extrinsic death-receptor and intrinsic mitochondrial pathways. Although endoplasmic reticulum (ER) stress is better described as an intrinsic, rather than extrinsic apoptotic signal, apoptosis induced by dysfunctional ER appears to rely on elements on both pathways, and thus their relative importance is unclear (Rutkowski & Kaufman, 2004).

The CLA preparations industrially produced and commercially available are used for scientific research as a mixture of equal proportions of the *c*9,*t*11 and the *t*10,*c*12 CLA isomers, accompanied by others in minor amounts. Thus, distinct effects on apoptosis have been reported for the CLA mixture and for each of these two isomers. In fact, the *t*10,*c*12 CLA isomer was reported as inducer of apoptotic genes expression, whereas *c*9,*t*11 CLA did not increase apoptosis in most of the reports (Kelley *et al.*, 2007). In addition, multiple studies have tried to identify the molecular pathways through which CLA induced apoptosis takes place. Still, much more effort should be expended to clarify whether CLA-induced apoptosis may be mechanistically related to CLA-induced body mass reduction (Serini *et al.*, 2009).

Furthermore, obesity frequently leads to changes in fatty acid metabolism with subsequent fatty infiltration of the liver, a phenomenon known as lipotoxicity (Wei *et al.*, 2007). This

pathophysiological condition comprises a wide spectrum of liver diseases that range from steatosis to non-alcoholic steatohepatitis and, ultimately, to fibrosis and cirrhosis (Browning & Horton, 2004). Fatty infiltration of the liver can arise either from increased hepatic uptake or synthesis of fatty acids, or decreased fatty acid excretion or catabolism. There is a link between obesity and ER stress (Özcan *et al.*, 2004), and recently it was suggested that fatty acids lipotoxicity in the liver involves impairment of ER-signaling proteins and c-Jun NH_2-terminal kinase (JNK) mediated apoptosis (Malhi *et al.*, 2006; Wang *et al.*, 2006a; Wei *et al.*, 2006; Zhang *et al.*, 2006). Indeed, saturated fatty acids play a role in lipotoxicity in liver cells and hepatocellular apoptosis is an important form of cell death, pushing forward the pathophysiology of human liver diseases (Wei *et al.*, 2006). The *fa*/*fa* Zucker genotype is an obese, leptin receptor-deficient model that exhibits hyperinsulinaemia without hyperglycaemia (Zucker & Zucker, 1961), non-alcoholic fatty liver disease (NAFLD), and hypertriglyceridaemia due to an increased hepatic production of very low density lipoproteins (Bray, 1977). Also, featuring these rats are adipocyte hyperplasia and hypertrophy (Marques *et al.*, 1998). For all of these reasons, the obese Zucker rat is an excellent model for obesity research (Kurtz *et al.*, 1989; Koteish & Diehl, 2001).

In the present study, obese Zucker rats were fed atherogenic diets formulated with fat from vegetable or animal origins, and supplemented with CLA as a combination of *c*9,*t*11 and *t*10,*c*12 CLA isomers in similar proportions. We looked for the effects of CLA, saturated fats and their interaction on apoptotic responses in white adipose tissue and on the hepatic lipotoxicity condition, which is inherent to steatotic Zucker rat liver. Finally, in order to elucidate the apoptotic mechanisms involved, we further assessed Fas death receptor, JNK pathway and ER-signaling proteins in rat liver.

7.2. EXPERIMENTAL PROCEDURES

7.2.1. Diet fats

The CLA oil, with 80% purity and similar percentages of *c*9,*t*11 and *t*10,*c*12 CLA isomers, was a generous gift from PharmaNutrients, Inc. (Gurnee, IL, USA). The remaining ingredients, apart from ovine fat, were purchased from Provimi Kliba, SA (Kaiseraugst, Switzerland) to manufacture the experimental diets.

7.2.2. Obese Zucker rats and experimental diets

The experimental protocol of this study was reviewed by the ethics commission of CIISA/FMV and approved by the Animal Care Committee of the National Veterinary Authority following the appropriate European Union guidelines (N. 86/609/EEC). Thirty two male *fa*/*fa* Zucker rats (Harlan Interfauna Iberica, S.L., Barcelona, Spain) aged 28 days were housed individually in stainless steel cages in an environmentally controlled room with temperature fixed at 22 ºC and a photoperiod of 12 h (lights on at 7.00 am). After arrival, rats were fed a commercial standard diet (Harlan Teklad Global Diets®2014, Harlan Interfauna Iberica, S.L.) without any sort of supplementation for one week. After this period, rats were assigned to four body weight matched groups, of eight animals each, and fed *ad libitum* semi-purified atherogenic diets and water for 14 weeks. The dietary treatments were based on AIN-93G standard formulation, modified accordingly to achieve atherogenic feeding regimens (Provimi Kliba, SA). Hence, four diets were enriched with 2% (w/w diet) of cholesterol (plus 0.5% (w/w diet) of sodium cholate to improve the cholesterol absorption) and with 15% (w/w diet) of fat which differ in fatty acid composition. Two groups were fed vegetable fat diets: P group - 11.25% (w/w diet) of palm oil plus 3.75% (w/w diet) of sunflower oil; PCLA group - 11.25% (w/w diet) of palm oil plus 2.53% (w/w diet) of sunflower oil plus 1.22% (w/w diet) of CLA mixture. The CLA mixture contained 40:40 of the *c*9,*t*11 and the *t*10,*c*12 CLA isomers. The other two groups received ovine fat instead of palm oil. The ovine intraperitoneal fat was obtained from lambs fed with pelleted dehydrated lucerne supplemented with 6% of a blend of sunflower and linseed oils (Jerónimo *et al.*, accepted), melted and then filtered to subsequent incorporation into the diets. The two diets supplemented with animal fat presented the following fat composition: O group - 11.25% (w/w diet) of ovine fat plus 3.75% (w/w diet) of sunflower oil; OCLA group - 11.25% (w/w diet) of ovine fat plus 2.53% (w/w diet) of sunflower oil plus 1.22% (w/w diet) of CLA mixture. The inclusion of 1.22% (w/w diet) of CLA mixture in dietary groups represents approximately 5% of the total fatty acids. The fatty acid profile and the composition of experimental diets are summarised in Table 5.1 (page 68) and Table 6.1 (page 83), respectively. Body weight and feed intake were measured twice a week. At the end of the experimental period, rats were fasted 12 h and killed by decapitation, under light isofluorane anesthesia. Tissues, as retroperitoneal fat and liver were excised, weighted, flash-frozen in liquid nitrogen, and stored at -80 ºC until analysis.

7.2.3. Histological analysis

Samples from liver and fat depot (approximately 100 mg) were fixed by immersion in 10% neutral buffered formalin (Merck, Darmstadt, Germany) for 24 h and processed for paraffin (Microscopy Histosec, Merck) embedding. Tissue sections (10 μm thick) were cut on a microtome from each of paraffin-embedded specimens and stained with hematoxylin and eosin for hepatic histological examination. Unstained sections were used for assessment of cell death in both retroperitoneal fat and liver.

7.2.4. TUNEL assays

Paraffin-embedded retroperitoneal fat and liver tissue sections were deparaffinised and pretreated with 20 μg/ml proteinase K (Roche Applied Science, Mannheim, Germany). An ApopTag® peroxidase *in situ* apoptosis detection kit (Serologicals Corp., Norcross, GA, USA) was used for transferase mediated dUTP-biotin nick-end labeling (TUNEL) staining. In brief, samples were treated with 3% of hydrogen peroxide to quench endogenous peroxidase activity. After adding the equilibration buffer, sections were treated with terminal deoxynucleotidyltransferase (TdT) and digoxigenin-dNTPs for 60 min at 37 ºC. Specimens were then treated with antidigoxigenin-peroxidase for 30 min at 37 ºC, colorised with 3,3´-diaminobenzidine (DAB) substrate, and counterstained with 0.5% methyl green. Finally, slides were rinsed, dehydrated, and mounted. A negative control was prepared by omitting the TdT enzyme to control for nonspecific incorporation of nucleotides or binding of enzyme-conjugate. The specimens were examined using a bright-field microscope (Olympus BX51, Tokyo, Japan) and the data expressed as the percentage of TUNEL-positive cells (×400). Apoptotic nuclei were identified by brown staining. Image analysis was a blind process to avoid bias.

7.2.5. Caspase-3-like activity

Caspase activation was determined in cytosolic protein extracts after harvesting and homogenisation of fat depot and liver samples in isolation buffer containing 10 mM Tris-HCl buffer, pH 7.6, 5 mM $MgCl_2$, 1.5 mM potassium acetate, 2 mM dithiothreitol, and protease inhibitor mixture tablets (Complete, Roche Applied Science, Manheim, Germany). General caspase-3-like activity was determined by enzymatic cleavage of chromophore *p*-nitroanilide (pNA) from the substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA, Sigma-Aldrich, St.

Louis, MO, USA). The reaction mixtures were incubated at 37 ºC for 1 h, and the formation of *p*-nitroanilide was measured at 405 nm using a 96-well plate reader.

7.2.6. Tissue protein isolation and immunoblot analysis

Four independent samples of each dietary group were processed for Western blot analysis. Liver samples were homogenised and total proteins were extracted and isolated. In brief, cells were lysed in ice-cold buffer containing 10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM potassium acetate, 1% Nonidet P-40, 2 mM dithiothreitol, and supplemented with protease inhibitor cocktail tablets (Complete, Roche Applied Science, Manheim, Germany) for 30 min, and then homogenised with 20 strokes in a loose fitting Dounce. The lysate was centrifuged at 3200 g for 10 min at 4 ºC and the supernatant recovered. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's specifications. Steady-state levels of Fas, p-JNK, p-c-Jun, ATF-6α, BiP and CHOP proteins were determined by Western blot analysis. Briefly, 100 μg of total protein extracts were separated on a 12% sodium dodecyl sulphate-polyacrylamide electrophoresis gel (SDS-PAGE). Following electrophoretic transfer onto nitrocellulose membranes, the immunoblots were incubated with 15% of H_2O_2 for 15 min at room temperature. After blocking with a 5% of milk solution during 1 h, the membranes were incubated overnight at 4 ºC with primary mouse monoclonal antibodies reactive to Fas, p-JNK (Santa Cruz Biotechnology, CA, USA), BiP (KDEL-Abcam, Cambridge Science Park, UK), or primary rabbit polyclonal antibodies to p-c-Jun, ATF-6α, CHOP (Santa Cruz Biotechnology), and finally with secondary anti-mouse antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories) for 3 h at room temperature. The membranes were processed for protein detection using SuperSignal substrate (Pierce, Rockford, IL, USA). β-Actin (Sigma-Aldrich, St. Louis, MO, USA) was used as loading control. The relative intensities of protein bands were analysed using the Quantity One Version 4.6 Densitometric Analysis Program (Bio-Rad Laboratories) and normalised to the respective loading control. When possible, samples were run in duplicate and/or repeated to confirm reliability of results.

7.2.7. Statistics

Data treatment was performed using the Statistical Analysis System, version 9.1 (SAS Institute, Cary, NC, USA). All data are reported as mean ± standard error of the mean (SEM).

The body composition parameters and apoptosis markers were analysed using the general linear model (procGLM) to perform a two-way analysis of variance (ANOVA) to look for CLA, fat and their interaction (CLA × fat) effects. Whenever CLA × fat interaction was found, a *post-hoc* Tukey's test was applied and statistical differences were marked by different letters. Statistical significance was set at $P<0.05$.

7.3. RESULTS

7.3.1. Apoptotic markers in the retroperitoneal fat

Table 7.1 shows the weight variation of final body, retroperitoneal fat and liver. In addition, the quantification of total lipids in the liver is shown. A CLA effect was observed for all above mentioned parameters ($P<0.001$), except for retroperitoneal fat weight. Reduced final body and liver weights were observed in groups fed CLA regardless the fat origin. Accordingly, the quantification of total lipids in the liver was reduced in PCLA and OCLA groups in relation to their counterparts. In contrast, neither CLA nor fat had any effect on retroperitoneal fat depots weight ($P>0.05$). In this study, the levels of cell death by apoptosis were evaluated using the TUNEL assay. Retroperitoneal fat had similar number of TUNEL-positive cells across the four dietary groups (Table 7.1, $P>0.05$). As a hallmark of downstream apoptotic events, caspase-3 activity was also determined in the adipose tissue. No significant differences were observed for caspase-3-like activity among the four dietary groups ($P>0.05$). Taken together, these findings corroborate the similar weights for retroperitoneal fat found in all dietary groups ($P>0.05$).

Table 7.1 Body composition parameters and markers of apoptosis in the retroperitoneal fat of obese Zucker rats fed four dietary treatments.

						Significance level		
	P	**PCLA**	**O**	**OCLA**	**SEM**	**CLA**	**Fat**	**CLA × Fat**
Body composition parameters								
Final body weight (g)	577	552	584	511	12.7	***	ns	ns
Retroperitoneal fat weight (g)	24.0	24.7	23.7	20.7	1.62	ns	ns	ns
Liver weight (g)	62.2	54.7	60.6	48.1	1.88	***	*	ns
Liver lipids (%)	21.9	18.9	23.5	19.2	0.75	***	ns	ns
Markers of apoptosis in retroperitoneal fat								
TUNEL assay (%)	7.29	5.60	6.96	7.08	1.340	ns	ns	ns
Caspase-3 activity (arbitrary units)	0.07	0.06	0.06	0.06	0.004	ns	ns	ns

Dietary treatments: P = palm oil; PLCA = palm oil + 1% CLA; O = ovine fat; OCLA = ovine fat + 1% CLA. Data were analysed by two-way ANOVA to look for CLA, fat and CLA × fat effects. Whenever the interaction CLA × fat was detected, a post-hoc Tukey´s test was applied ans statistical differences were marked by different superscript letters. Significance levels: not significant (ns), $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

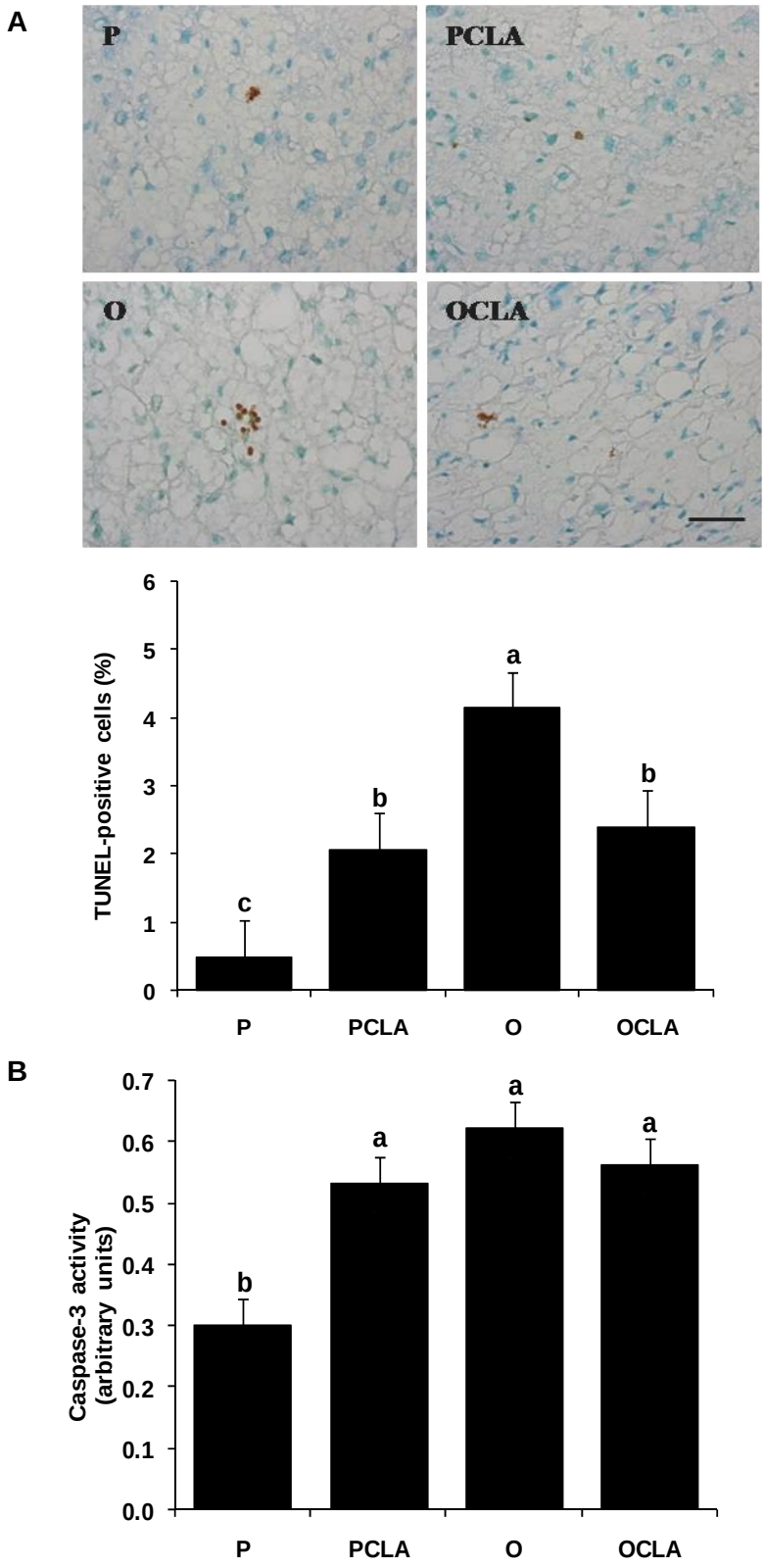

Figure 7.1 Percentage of TUNEL-positive cells at a high power field (×400) (A) and caspase-3-like activity (B) in the liver of obese Zucker rats fed the various diets (P = palm oil diet; PCLA = palm oil + 1% CLA diet; O = ovine fat diet; OCLA = ovine fat + 1% CLA diet). Representative images of TUNEL staining, with apoptotic cells identified by a condensed nucleus with brown staining, are shown (scale bar: 100 μm). Data are expressed as mean ± SEM, with *n*=8 rats *per* group. A CLA × fat interaction effect was detected in both parameters by two-way ANOVA ($P<0.01$). Bars with different letters are significantly different by *post-hoc* Tukey's test ($P<0.05$).

7.3.2. Apoptotic markers in the liver

Hepatic steatosis is currently recognised as a characteristic of *fa*/*fa* mutant rats. Indeed, livers from all groups presented severe steatosis with minimal portal mononuclear infiltrate (see histological images in Figure 7.1A). Levels of apoptosis were lower in the liver of the palm oil group relatively to the other dietary treatments (Figure 7.1A, $P<0.05$). This observation was further corroborated by the caspase-3 activity assays (Figure 7.1B). An interaction between CLA and fat was observed ($P<0.05$). CLA induced caspase-3 activity in vegetable fat group ($P<0.05$) but O and OCLA groups remained unchanged (Figure 7.1B, $P<0.05$).

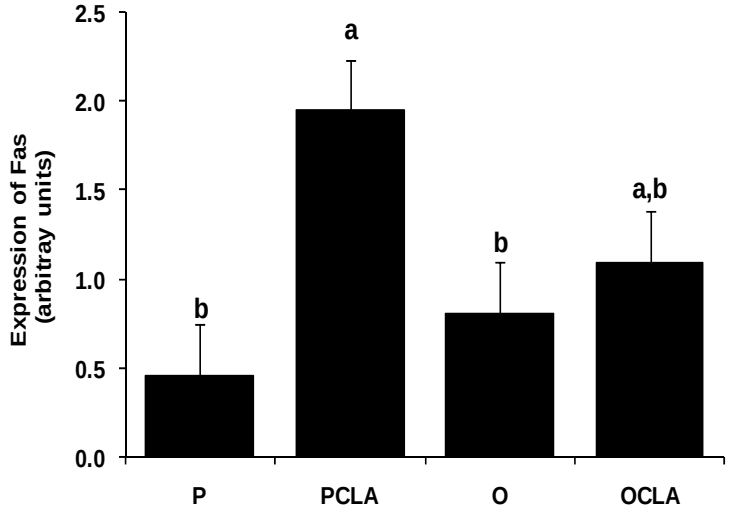

Figure 7.2 Fas protein expression in the liver of obese Zucker rats fed the various diets (P = palm oil diet; PCLA = palm oil + 1% CLA diet; O = ovine fat diet; OCLA = ovine fat + 1% CLA diet). Data are expressed as mean ± SEM, with n=4 rats *per* group. A CLA × fat interaction effect was detected in Fas by two-way ANOVA ($P<0.05$). Bars with different letters are significantly different by *post-hoc* Tukey's test ($P<0.05$).

For both parameters, an interaction CLA × fat was observed ($P<0.01$). Interestingly, CLA had a contrasting effect on hepatocytes apoptosis, according to the dietary fat origin. In the presence of a vegetable fat as palm oil, CLA increased TUNEL-positive cells and caspase-3 activity levels ($P<0.05$). In contrast, CLA reduced the number of TUNEL-positive cells in rats fed a diet with animal fat origin ($P<0.05$). We next investigated the expression of the death receptor Fas in the liver for all dietary groups (Figure 7.2). An interaction between CLA and fat was observed ($P<0.05$). CLA induced Fas expression in vegetable fat group ($P<0.05$) but O and OCLA groups remained similar ($P>0.05$). Additionally, no changes were observed when comparing groups with different fat types, with or without CLA ($P>0.05$). The JNK signaling pathway was subsequently considered and variations of p-JNK and p-c-Jun proteins are represented in Figure 7.3. CLA increased p-JNK expression ($P<0.05$), independently of fat origin.

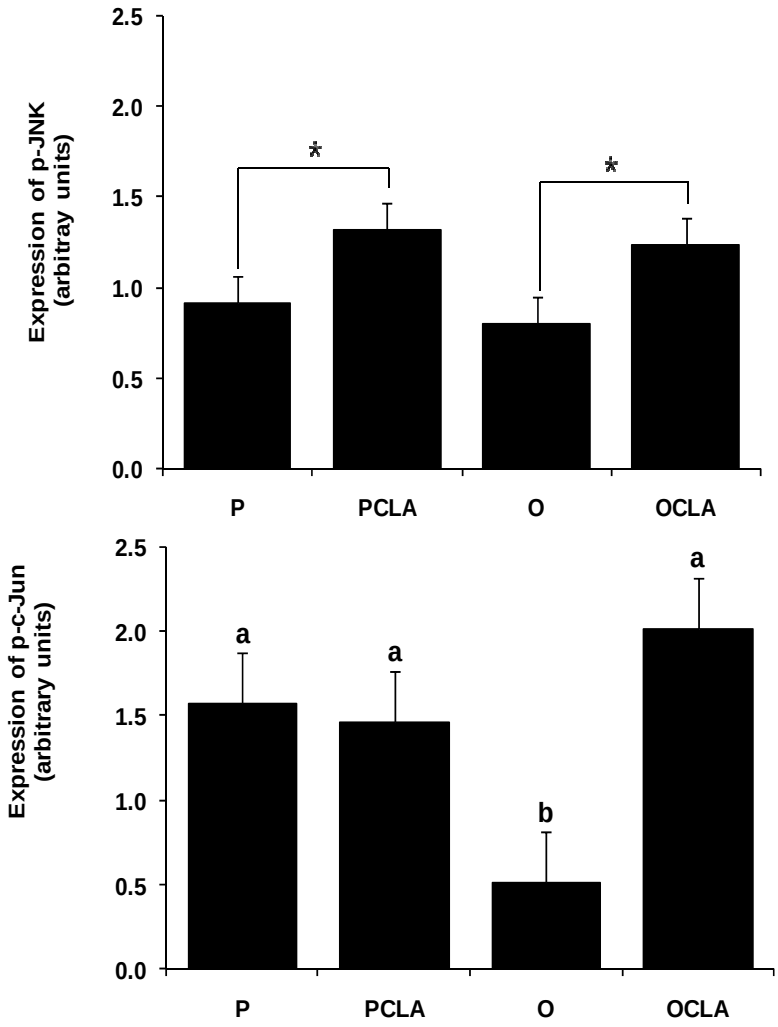

Figure 7.3 p-JNK and p-c-Jun protein expression in the liver of obese Zucker rats fed the various diets (P = palm oil diet; PCLA = palm oil + 1% CLA diet; O = ovine fat diet; OCLA = ovine fat + 1% CLA diet). Data are expressed as mean ± SEM, with *n*=4 rats *per* group. ***** - A significant CLA effect was detected in p-JNK by two-way ANOVA ($P<0.05$). A CLA × fat interaction effect was detected in p-c-Jun by two-way ANOVA ($P<0.01$). Bars with different letters are significantly different by *post-hoc* Tukey's test ($P<0.05$).

No effects of fat (vegetable or animal) or CLA × fat interaction were observed ($P>0.05$). In addition, p-c-Jun was influenced by CLA × fat interaction ($P<0.01$), being reduced in the ovine fat group without CLA ($P<0.05$). Expression of the ER-signaling proteins ATF-6α, BiP and CHOP were also investigated (Figure 7.4). An interaction CLA × fat was observed for the three ER markers ($P<0.01$). CLA supplementation reduced ATF-6α protein expression in vegetable fat diet ($P<0.05$). Conversely, CLA induced ATF-6α expression in animal fat diet ($P<0.05$). That is, the ovine fat group had a lesser expression of ATF-6α than the palm oil group ($P<0.05$). In contrast, the OCLA group had a higher expression of ATF-6α than the PCLA group ($P<0.05$). No differences were found in BiP expression between P and PCLA groups, suggesting that CLA had no part on differential activation of BiP protein facing a vegetable fat diet ($P>0.05$).

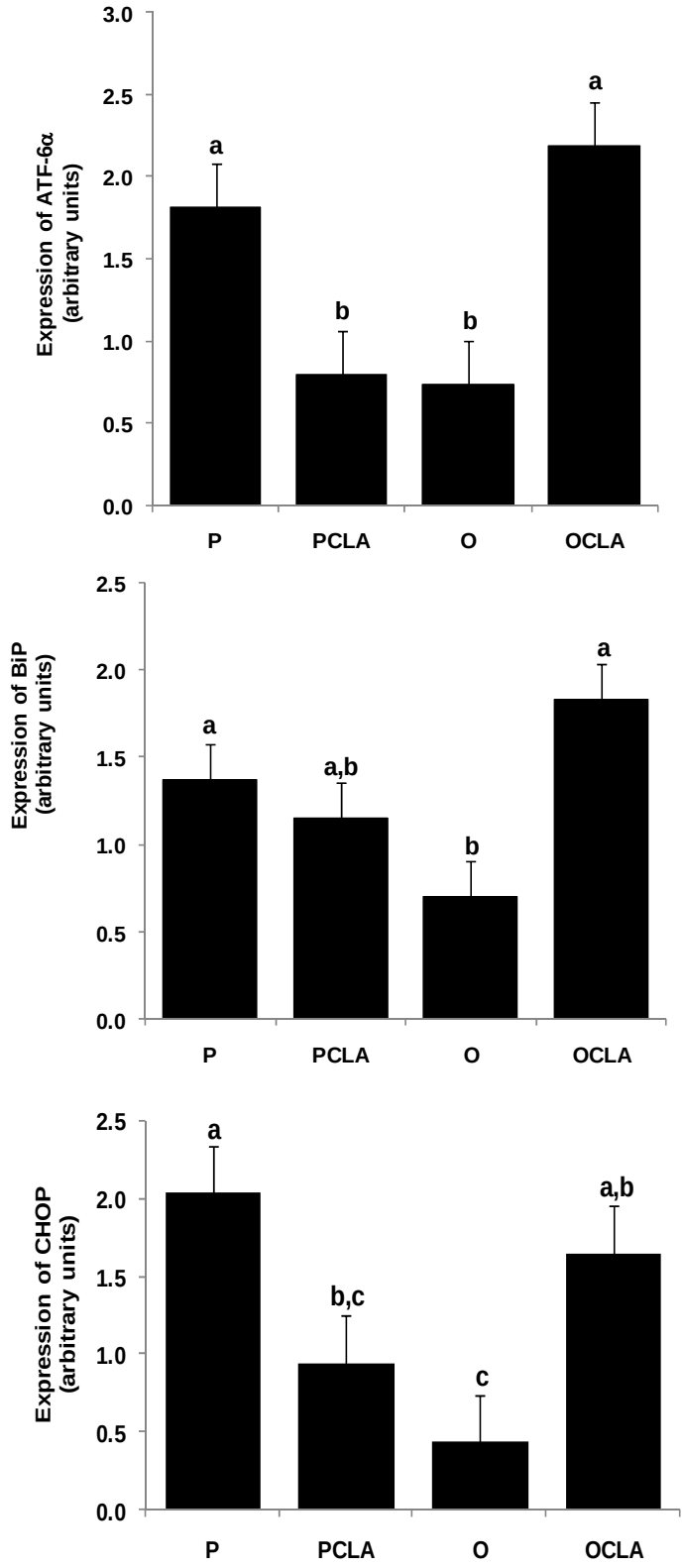

Figure 7.4 ATF-6α, BiP and CHOP protein expression in the liver of obese Zucker rats fed the various diets (P = palm oil diet; PCLA = palm oil + 1% CLA diet; O = ovine fat diet; OCLA = ovine fat + 1% CLA diet). Data are expressed as mean ± SEM, with n=4 rats *per* group. A CLA × fat interaction effect was detected by two-way ANOVA (P<0.01). Bars with different letters are significantly different by *post-hoc* Tukey's test (P<0.05).

The same cannot be applied to the pair O/OCLA, as the addition of CLA promoted higher BiP expression ($P<0.05$). Moreover, no changes were observed between PCLA and OCLA groups ($P>0.05$). Without CLA, the palm oil group had more BiP expressed than the ovine fat group ($P<0.05$). The variations found for CHOP expression across the dietary treatments are identical to those of ATF-6α protein expression in what relates to CLA × fat outcomes (Figure 7.4). The ovine fat group (O) *per se* decreased the expression of this protein ($P<0.05$). However, groups supplemented with CLA (PCLA and OCLA) had no statistical changes ($P>0.05$).

7.4. DISCUSSION

The present study yields results that cast doubt on the benefits of CLA in obese Zucker rats, as far as visceral fat reduction concerns. This work contrasts with previous studies in obese mice with different genetics suggesting that dietary CLA, either as a mixture or solely the *t*10,*c*12 CLA isomer, can decrease body fat through adipocyte apoptosis (Tsuboyama-Kasaoka *et al.*, 2000; Miner *et al.*, 2001; Hargrave *et al.*, 2002; Hargrave *et al.*, 2004). In our study and others (Sisk *et al.*, 2001; Inoue *et al.*, 2006), the ingestion of 1% of combined *c*9,*t*11 and *t*10,*c*12 CLA isomers induced no reduction on adipose depots weight in obese Zucker rats. The ultimate manifestations of apoptosis were here assessed by TUNEL assay and caspase-3-like activity in retroperitoneal fat depots. TUNEL positivity has been controversial (Zinszner *et al.*, 1998) as it can be observed in both necrosis and apoptosis processes, and false positives are common. In this study, the assessment of programmed cell death was complemented by the caspase-3 activity assay. No variations among dietary groups were found for both parameters, which is in line with similar retroperitoneal fat weight data. The obese genotype appears to overlap CLA reducing weight properties (Sisk *et al.*, 2001; Plourde *et al.*, 2008). Indeed, at the end of our experiment, rat body weight was reduced by CLA but not at visceral fat depots (including epididymal fat, data not shown) expenses. This can be justified by either reduction on liver weight or a slight, but statistically significant, decrease in food daily intake in rats fed CLA (23.8 to 22.4 g/day). Still, the subcutaneous fat was not weighted in this work due to methodological constraints. This former adipose depot might also contribute to the reduction on final body weight of rats fed CLA.

In this study, all rat groups presented hepatic steatosis, inherent to the leptin impairment function (Serkova *et al.*, 2006) and insulin resistance (Oda *et al.*, 2008), and likely intensified

by the consumption of atherogenic diets. Hepatic steatosis is defined as an accumulation of lipids to 5-10% of the liver by weight (Cairns & Peters, 1983) and all dietary groups had around 20% of total lipids in the liver. Albeit, dietary CLA significantly reduced liver weight of obese Zucker rats, in accordance to Sisk *et al.* (2001) findings. Total lipids in the liver were also reduced by CLA in this experimental trial, regardless the fat origin. The reduction of hepatic lipids by CLA might be explained by a decrease in gene activation of lipogenic enzymes, such as fatty acid synthase and stearoyl-CoA desaturase by inhibition of sterol regulatory element-binding protein 1 (SREBP-1) (Park *et al.*, 2001b; Lin *et al.*, 2004; Miyazaki *et al.*, 2004) which affects triacylglycerol accumulation inside the hepatocytes. Alternatively, CLA supplementation might induce fatty acid oxidation in the liver (Rahman *et al.*, 2001). At first glance, these are positive effects for CLA, although the histological pattern of lipotoxicity and steatosis was not reversed. In fact, previous studies claimed that among the possible adverse effects of CLA supplementation are the worsening of insulin resistance, enhance of total lipids in the liver and even, induction of hepatic steatosis (Sanders *et al.*, 2004; Poirier *et al.*, 2005; Wendel *et al.*, 2008; Ramos *et al.*, 2009).

Interestingly, CLA had a contrasting effect on cell death in the liver according to the dietary fat. CLA increased hepatocyte apoptosis in rats fed palm oil diet but reduced the apoptotic responses in rats fed animal fat. This may be likely justified not only by CLA addition but also by the fatty acid composition of the matrix diets and by the interaction between these two factors. Free fatty acids, as palmitic acid (16:0), can induce lipotoxicity and cell apoptosis in different cell cultures (De Vries *et al.*, 1997; Kharroubi *et al.*, 2004; Jeffrey *et al.*, 2008; Peter *et al.*, 2008). This explanation is in agreement with our findings. Apoptosis levels were enhanced in the liver of rats fed palm oil with CLA, being the palmitic acid 2.1% higher in this diet. In addition, oleic acid (18:1*c*9) has been shown to prevent apoptotic cell death induced by the *t*10,*c*12 CLA isomer in rat hepatoma cells (Yamasaki *et al.*, 2008). Knowing that diets with CLA had higher contents of *t*10,*c*12 CLA isomer and lower percentages of oleic acid, the increased levels of apoptosis in the palm oil group with CLA may be justified by the lack of ameliorative effect of oleic acid on apoptosis by *t*10,*c*12 CLA isomer. This hypothesis, however, fails to justify the lower level of cell death found in the ovine fat group with CLA, which is discussed below.

The increase of Fas death receptor expression in the liver by CLA mixture, when added to a diet saturated in palm oil, confirms the involvement of the extrinsic pathway of apoptosis. Fas-mediated hepatocyte apoptosis can lead to up regulation of pro-inflammatory chemokines

(Faouzi *et al.*, 2001) and contribute to inflammation. The histological picture of livers from the palm oil group with CLA (and from the other dietary treatments, too) allowed us to conclude the absence of an aggravated inflammatory process. One possible explanation may rely in the dose-response of cell death. In fact, about 10 to 15% of hepatocytes were shown to undergo apoptosis before the onset of neutrophil induced injury (Lawson *et al.*, 1998). Yet, our final cell death counting was utmost 5% of hepatocyte apoptosis for all dietary groups.

When comparing rats fed with ovine fat, the group with CLA supplementation had lower levels of apoptosis, with higher activation of JNK pathway and ER-signaling proteins. Curiously, the variations for ER-signaling proteins and cell death counting were consistent across the four groups; an augment of apoptosis in palm oil group with CLA was accompanied by reduction patterns of ATF-6α, BiP and CHOP proteins and the inverse was observed in ovine fat group with CLA. In this regard, CLA supplementation appeared to restore ER protein secretion and activate the JNK pathway, suggesting a counter-adaptive response of survival that may explain the reduced levels of apoptosis in this group (Herdegen *et al.*, 1997). Our study lacks evidence of ER stress in ovine fat group with CLA. The most reduced levels of apoptosis were observed with palm oil alone, and matched the same amount of ER-signaling proteins as seen in ovine fat group with CLA. Any comparison between our results and others, mostly from *in vitro* studies, should be handled with caution. *In vitro*, it is easier to track chronologically the events and manifestations of programmed cell death (*e.g.* with hours apart). However, our experiment lasted for 14 weeks mimetising chronic exposure of obese Zucker rats to saturated diets with or without CLA.

Finally, the diets at study were enriched in saturated fatty acids and cholesterol. Overall, the palm oil had more palmitic, oleic and linoleic acids, whilst ovine fat had much higher percentages of estearic (18:0), α-linolenic (18:3*n*-3) and *trans* fatty acids, mainly vaccenic acid (18:1*t*11) but also others (see Table 5.1, page 68). Even though the vaccenic acid is metabolised into *c*9,*t*11 CLA isomer (Lock *et al.*, 2004) and for this reason, has been considered as beneficial or neutral, the biological properties of *trans* fatty acids of ruminant fat origin are poorly exploited and still believed, as unhealthy lipids (Willet, 2006; Prates & Bessa, 2009). In this study, besides 18:1*t*11, animal fat provided more *trans* fatty acids, including *trans* 18:1 positional isomers from *t*6 to *t*12 and several 18:2 *trans* isomers. Although the biological effects of most of these isomers remain elusive, some data indicate elaidic acid (18:1*t*9) and linoelaidic acid (18:2*t*9,*t*12) as inducers of early and late apoptosis

stages (Zapolska-Downar *et al.*, 2005). ER-signaling protein modulation by CLA in rats fed ovine fat can probably relate to the higher total CLA content (6.5%) due to the endogenous amount of CLA (1.9%) in the ovine fat, facing an overload of *trans* fatty acids (see Table 5.1, page 68).

7.5. CONCLUSIONS

To the best of our knowledge, this is the first report on the influence of feeding CLA and saturated fat diets, from vegetal and animal origins, in cellular apoptosis of white adipose tissue and liver using an obese research model. In addition to the presence of CLA, our results underline the importance of fatty acids as diet components with discriminatory roles on apoptosis molecular responses in the liver. In fact, the higher levels of apoptosis found in palm oil diet with CLA, appear dependent on the extrinsic pathway, through Fas up regulation. In contrast, the ovine fat group with CLA showed less apoptosis but higher secretion of ER-signaling proteins and activation of JNK pathway, suggesting an adaptive response of cell survival. Forthcoming *in vitro* studies focusing on the induction of apoptosis and related molecular pathways by individual dietary fatty acids, including CLA, are strongly encouraged.

Acknowledgments

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CHAPTER 8 General discussion and future perspectives

Scientific advances during the last century helped in the discovery of new drugs promoting huge advances in human health care services, thus, enabling a longer life expectation. However, lifestyles changed, industrialisation greatly increased, agricultural practices declined, and the improvement of economic conditions with greater food accessibility have resulted in a more sedentary population and in a dramatic shift in the composition of the diets consumed (WHO, 2003). These factors have increased chronic pathologies, such as diabetes, cardiovascular diseases, and some cancers, most of them related with an abnormal accumulation of adiposity. As it is difficult to reach nutritional targets for dietary fat consumption, the use of natural compounds with bioactive properties, able to produce functional foods, constitutes an interesting strategy to attenuate and prevent obesity incidence. CLA has been studied mainly towards this objective. The studies reported in this thesis aimed to elucidate the biological effects of CLA when administrated to distinct animal models involving different approaches as adipocyte metabolism, tissue fatty acid deposition, permeability of celullar membranes and apoptosis.

8.1. CLA EFFECTS ON THE NORMAL-WEIGHT WISTAR RAT

There is much evidence that *c*9,*t*11 and *t*10,*c*12 CLA isomers exert distinct biological effects. For example, in several animal feeding studies, the *t*10,*c*12 isomer was responsible for a reduction in body fat weight (see review Park & Pariza, 2007b). However, this isomer has been held responsible for a pro-diabetic effect in both humans and mice (Riséurs *et al.*, 2002; Roche *et al.*, 2002). Facing this, chapters 3 and 4 were designed to examine the effect of these two individual CLA isomers. For this purpose, a reference strain that presents a normal growth development with no genetic predisposition for any pathology was selected, the Wistar rat. This experimental model aims to mimetise a healthy normal weight subject that consumes CLA supplements. Palm oil is currently the second largest edible oil in terms of global production, after soybean oil (Edem, 2002), and all experimental diets contained palm oil in a refining form. Today, as legislation obliges the divulgation of TFA, mainly originated from partially-hydrogenated oils, on the nutritional label of commercial food products, driving many manufacturers to replace them with natural sources of saturated fat, as palm oil. It is

frequently found in margarines, shortenings, ice creams, cocoa substitutes in chocolate, just to name a few examples.

The dietary supplementation of CLA isomers, individually or in combination, to Wistar rats did not result in different growth performances, unaffecting the weight of several organs, including the main adipose depots. Also, the serum profile of lipids and adipokines were not affected, except for triacylglycerols. Nothwithstanding, the morphology of adipocytes changed in the presence of *c*9,*t*11 CLA isomer, in both epididymal and retroperitoneal adipose depots. To the best of our knowledge, it was reported for the first time an increase of adipocytes area due to *c*9,*t*11 CLA isomer. It is important to highlight that the CLA mixture did not affect body fat composition or adipocyte morphology, suggesting a compensation between *c*9,*t*11 and *t*10,*c*12 CLA isomers effects. Remaining to elucidate are the mechanisms involved, although the parallel increase of serum triacylglycerols suggests consequences at the level of lipid metabolism, such as an increase in lipogenesis rate. In the same trial, other research approach was followed to further explore triacylglycerols synthesis, concretely, in what glycerol metabolism concerns. The effect of CLA on the permeability of kidney proximal tubules membranes to glycerol was an innovator study that resulted in novel important findings. CLA mixture promoted an increase in glycerol permeability through the kidney vesicles membranes and not by protein-mediated transport. This result may be attributed to an increase in lipid bilayer fluidity through the incorporation of CLA isomers in the membrane re-arrangement. The absence of differential responses in protein-mediated transport suggest that CLA did not affect the expression and/or activity of aquaglyceroporin channels, namely AQP7. Kidney is an important organ on the reabsorption pathway of glycerol re-directing it to the liver. Once in the liver, glycerol can participate in lipogenesis or can be converted to glucose. Still, this potential pathway for triacylglycerols increase by the CLA mixture is not consensual with the effective augmentation of triacylglycerols only by the *c*9,*t*11 isomer. Summing up, it was not observed any effect on body composition of *c*9,*t*11 and *t*10,*c*12 CLA isomers separately or as a mixture.

8.2. CLA EFFECTS ON THE OBESE ZUCKER RAT

Inconsistent anti-adipogenic effects of CLA have been found in humans and in different animal models. Up to date, no human trials have tried to disclosure the efficacy of CLA supplementation on body fat loss in obese subjects with a well characterised genetic

background. Additionally to the environmental factors, some studies have indicated that genetic factors account for a substantial portion of human adiposity (Barness *et al.*, 2007). Among many, human mutations in the leptin gene (Montague *et al.*, 1997) and leptin receptor (Clement *et al.*, 1998) are well characterised, resulting in a severe obesity phenotype, which indicates that leptin is critical to body weight regulation. The *fa*/*fa* Zucker rat is a strain of laboratory animal descendent from the wild model rodent *Rattus norvegicus* (Zucker & Zucker, 1961), used often to study genetic obesity problems. This animal model develops morbid obesity, through the *fa*/*fa* mutation in the extracellular domain of the leptin receptor, which inhibits completely leptin action. This leads to an appetite increase promoting severe insulin resistance (Bray, 1977). According to the same author, obese Zucker rats do not have pancreatic β-cells defect, and do not progress to insulin deficiency similar to human type 2 diabetes. In the present thesis, chapters 5, 6 and 7 were focused on CLA effects in obese Zucker rats fed an hyperlipidic (high fat) and atherogenic (high cholesterol) diet. This trial aimed to mimic an obese human sub-population with severe metabolic problems that consumes CLA supplements. However, CLA supplementation accounts for a small part of the total fatty acids ingested through the diet. So, the original hypothesis was that CLA could have different behaviours when supplemented to diets with vegetable or animal saturated fats. Most of the saturated fat intake is attributed to edible fats from ruminant origin (Givens, 2005). Both palm oil and ovine fat present high saturated fat content but different fatty acid composition. Palm oil is rich in palmitic acid while ovine fat possesses more stearic acid and TFA due to the rumen metabolism.

The results reported in this thesis revealed interesting effects of CLA and source of dietary fat, separately, but also effects denoting an interaction between CLA and fat source. The administration to rats of ovine fat rich in ruminant minor isomers, and an accurate separation methodology, allowed for the first time a detailed characterisation of fatty acid profile in rat tissues. It was notorious the specific incorporation of the various fatty acids in liver and muscle.

After the 14th week trial, Zucker rats exhibited massive obesity which was prevalent in the abdominal region. Despite having great amounts of subcutaneous fat, just epididymal and retroperitoneal adipose depots (visceral fats) were in fact weighted and analysed. The adipose tissue located in the visceral compartment, in contrast to the subcutaneous fat, contributes more actively to a pro-inflammatory state and lost of insulin sensitivity in obese subjects (Dolinková *et al.*, 2008). In the present work, the fatty acid profile of both epididymal and

retroperitoneal fat depots were quite similar. The adipokines quantification was performed in the serum but a differential adipokines secretion from different fat depots, and even liver, could be expected. Due to the leptin receptor mutation, it was not surprising the high levels of leptin found in the serum of *fa*/*fa* Zucker rats. The leptin action is based on the signaling in the hypothalamus. In there, a cascade of events is initiated, comprising the simultaneous decrease in appetite and insulin secretion with subsequent increase in metabolic rate (Savage & O´Rahilly, 2002). Naturally, one major consequence of leptin deficiency action is overfeeding (Tritos & Mantzoros, 1997). Beck *et al.* (2003) described that lean and obese adult Zucker rats daily feed 18 g and 23 g, respectively. Regarding the two experimental trials presented in this thesis, the Wistar rats consumed daily 22 g, in average, while obese Zucker rats consumed 23 g. Converting these values to energy, 81 and 93 kcal/day, respectively, the difference is still not to large. Many more physiologic discrepancies are reported between lean and obese Zucker rats. For example, Oana and co-workers (2005) showed that levels of insulin, adiponectin, triacylglycerols and free fatty acids in the plasma of obese Zucker rats were higher than that of lean rats but glucose levels were not significant different. Nevertheless, the evaluation of the genetic influences on CLA action was not a goal of the present thesis, and foremost important, the two trials had completely independent designs thus disabling their direct comparison.

There were positive and negative metabolic effects of CLA in the Zucker rat trial. In fact, CLA increased adiponectin and decreased PAI-1 serum levels, as well as, alleviated hepatic steatosis by means of hepatic lipids reduction. In contrast, the biochemical serum profile was changed with increased levels of total and LDL-cholesterol. The presence of two disctinct saturated fats, of vegetable and animal origins, in the diet also influenced several important adipokines, regardless CLA supplementation. In overall, the clinical state of the animals was aggravated by the ingestion of animal fat, regarding insulin resistance and inflammation. Ovine fat encloses a ruminant fatty acid profile highly consumed in Western societies, and these results reinforce the non-beneficial effects of this fat type.

The apoptotic studies presented in this thesis were pioneer where two main questions were addressed. Firstly, we asked if CLA can promote loss of body fat through adipocyte apoptosis, despite the genetics of the experimental model. The results for retroperitoneal depot revealed no differences in the cell death number among groups, which is consistant with similar fat depot weights. Secondly, facing a lipotoxicity condition observed in the steatotic

liver and aggravated by atherogenic diets, we looked for the influence of CLA supplementation and saturated fats from different sources in apoptotic molecular responses. All animals presented about 20% of hepatic lipids, which is indicative of an acute lipototixity process in the liver. Hepatocyte apoptosis is recognised as an important mechanism contributing to the progression of NAFLD (Feldstein & Gores, 2005). The inhibition of apoptosis represents a therapeutic target to treat human liver diseases (Ribeiro *et al.*, 2004). The apoptotic pathways exploited revealed a differential influence of dietary fatty acids according to their origin. The higher levels of apoptosis found in palm oil diet with CLA appeared dependent on the extrinsic pathway through upregulation of the cell death receptor Fas. In addition, Fas-mediated hepatocyte apoptosis can lead to the upregulation of pro-inflammatory chemokines (Faouzi *et al.*, 2001) and therefore, may contribute to inflammation. Contrarily to this hypothesis, the serum pro-inflammatory chemokines levels were kept unchanged in palm oil groups. The ability of apoptotic hepatocytes to synthesise peptide mediators that recruit inflammatory cells violates one supposed dogma of apoptosis. The basic principle behind the signalling events during apoptotic cell death is that once the point of no return is reached, that is, activation of downstream caspases cascade (which comprises caspase-3) a rapid breakdown of the cell is organised (Green, 2000). This happens without eliciting an inflammatory response and avoiding the waste of energy for useless repair processes. Contrarily, the ovine fat group with CLA showed less apoptosis than ovine fat groups alone, but higher secretion of ER-signaling proteins (ATF-6α, CHOP and BiP) and activation of JNK pathway (p-JNK and p-c-Jun), suggesting an adaptive response of cell survival. Additionally to ER-stress, mitochondrial pathway is another apoptotic route associated with hepatocytes apoptosis (Ribeiro *et al.*, 2004) but that was not exploited in the present work. In order to do so, it should be assessed the Bcl-2 family members, in particular, the balance between the expression of the anti-apoptotic protein Bcl-2 and the pro-apoptotic proteins Bax, Bad and Bid.

Contrarily to the Wistar rat trial, in the obese Zucker experiment the administration of atherogenic diets with 2% cholesterol, allowed us to look for possible alterations in aorta microvasculature. However, atheroma plaques were not observed for any dietary group. In fact, rodents do not represent a good model for atherogenesis studies (Narayanaswamy *et al.*, 2000). Atherosclerosis is not a natural disease in rodents and when dietarily induced with atherogenic diets, the resultant lesions are not consistent (Vesselinovitch & Wissler, 1968). Species type, body size and average blood pressure are key factors for atherosclerosis

development, which makes rabbit the most suitable species to perform these researches (Narayanaswamy *et al.*, 2000).

8.3. MAIN CLA EFFECTS ON BOTH MODELS

An overview of the main results obtained in the two rat trials are presented in Table 8.1, classified into deleterious, neutral and beneficial effects. The few differences observed in Wistar rat trial were due to the *c*9,*t*11 isomer and CLA mixture. CLA mixture affected the cell membrane permeability facing glycerol fluxes. The obese Zucker rats were affected by CLA supplementation but some effects were dependent on the saturated fat type available in the diet. It is important to underline that both *in vivo* studies did not provide any evidences of anti-adipogenic CLA action. Therefore, the present work questions the usefulness of CLA isomers to prevent or reduce obesity in subjects from Western societies, where the intake of animal saturated fats is prevalent.

Table 8.1 Overall findings of CLA and saturated dietary fat effects in both animal models.

Main effects in Wistar rat experiment		
deleterious	**neutral**	**beneficial**
↑ serum triacylglycerols by *c*9,*t*11	= daily intake	n.d.
↑ adipocyte size by *c*9,*t*11	= final body weight	
↑ permeability to glycerol by CLA	= organs weight	
	= adipose depots mass	
	= serum leptin and adiponectin	
	= permeability to water	
Main effects in obese Zucker rat experiment		
deleterious	**neutral**	**beneficial**
↑ serum total and LDL-cholesterol by CLA	= adipose depots mass	↓ daily intake by CLA
↑ serum pro-inflamatory citokines by ovine fat	= adipocytes apoptosis	↓ final body weight by CLA
↑ serum insulin resistance by ovine fat		↓ liver weight by CLA
↑ serum leptin by ovine fat		↓ liver lipids by CLA
		↓ muscle lipids by CLA
		↓ serum glucose by CLA
		↓ serum PAI-1 by CLA
		↑ serum adiponectin by CLA
		↓ hepatocytes apoptosis by CLA in ovine fat diet

CLA = mixture of similar proportions of *c*9,*t*11 and *t*10,*c*12 isomers; n.d. – not detected.

8.4. POSSIBLE EXTRAPOLATIONS TO HUMANS

In the present work, the rat was the chosen model to test CLA biological effects. The rat is a highly valuable model for the investigation of cardiovascular diseases, metabolic disorders (*e.g.* lipid metabolism, diabetes), cancers, renal disfunctions, neurologic pathologies, and so many other diseases (Van Zutphen *et al.*, 2001). As any model used for human health applications, laboratory animal science has important limitations in the extrapolation of the results. This extrapolation should consider different genetics, metabolic rates, polymorphisms, among other factors. The amount of CLA administrated to rats was 1% of diet weight, the most used dose in research works, which means 220-230 mg of CLA *per* day. This thesis presents, for the first time, the estimation of CLA daily intake for the Portuguese population, based on the composition of CLA-rich products ingested by regular diet (chapter 1). The value found, 74 mg/day, is much lower than the daily value proposed for humans to achieve beneficial effects, which is 0.8-3 g/day (Ip *et al.*, 1994; Parish *et al.*, 2004). This means that, according to animal studies, to reach possible protective effects, the intake of CLA supplements has to be considered. In comparison, CLA dietary supplements marketed for weight loss constitute an intake of 2-4 g/day (Pharma Nord, 2009). Most of the CLA products sold as dietary supplements contain 60-90% of CLA, predominantly, *c*9,*t*11 and *t*10,*c*12 isomers, in the form of either free fatty acids or triacylglycerols. The wide ranges of supplements CLA dosages and compositions, introduce inconsistencies and add more difficulties in the interpretation of CLA biological effects in humans. According to a recent review, approximately 50% of the studies report losses of fat mass after CLA intake in normal, overweight, and obese subjects (Plourde *et al.*, 2008). But new and stronger evidences are needed to consider CLA as supplement for fat loss agent in humans (Plourde *et al.*, 2008). In terms of other CLA properties, some human studies revealed adverse CLA effects. Recent evidence suggests that CLA may actually decrease HDL-cholesterol levels and increase markers of inflammation in the short term (Steck *et al.*, 2007). In obese men with metabolic syndrome, CLA worsened the insulin resistance (Risérus *et al.*, 2002, 2004). As being so, Stachowska and Chlubek (2009) advise that exist groups of "increased risk" for CLA use: subjects with diabetis, intolerance to glucose and a coronary thrombosis.

Nevertheless, it is of relevance that, in some European countries, CLA-enriched foods are available, as for example yogurts, milk and margarine. The foodstuff containing CLA is classified as a novel food according to the Novel Food Regulation (N. 258/97/EC), as it is "a

food or food ingredient consisting of or isolated from plants and food ingredients isolated from animals» and was not obtained by «traditional propagating or breeding practices and having a history of safe food". In 2008, CLA was recognised by the Food and Drug Administration (FDA) as Generally Regarded as Safe (GRAS). Owing to this status, in the USA, CLA can now be added, for instance, to flavoured milks, soya milks, milk based meal replacements, yoghurts, fruit juices.

In a general overview, beneficial and adverse effects of CLA were obtained in both trials described in this thesis. CLA supplements should be used carefully since the results obtained support the hypothesis that the diet composition has a significant impact on the metabolic processes studied so far. These results demonstrate the ability of different dietary matrixes to exert discrepant effects in several biochemical and metabolic pathways. The ability of fatty acids to modulate inflammation is particularly important as an increased number of diseases related to obesity were shown to have an underlying inflammatory processes. However, the use of CLA, as nutrient or drug, needs to ensure that excludes the risk factors associated with other disorders.

8.5. FUTURE PERSPECTIVES

At the end of this thesis some important work guidelines are suggested:

- The results presented in this thesis enphasise the need to further study biological and clinical interactions between different CLA isomers and the other fatty acids provided by the diet. It would be interesting to test *in vitro*, as a preliminary study, combinations between CLA isomers and palmitic acid, stearic acid, and TFA, for example. For that purpose, 3T3-L1 adipocytes can be used as a model, and molecular biology assays can be conducted to investigate the lipid metabolism and apopotic pathways.
- Regarding the possible health benefits for humans, it is necessary to continue to explore CLA effects according to different health status. CLA supplements are available for a general population that possesses different genetic backgrounds and are exposed to a variety of environmental factors that can influence the biological activity of CLA isomers.
- The permeability study conducted in the kidney vesicles from the Wistar rat trial, led us to investigate more deeply how CLA affects the glycerol metabolism. Recently, it was

reported that these aquaporin mediated-transport have a crucial role in lipogenesis (Hibuse *et al.*, 2006). Therefore, an ongoing work is already being performed on membrane permeability to glycerol of adipose cells from the obese Zucker rats. Specifically, we intend to investigate how CLA can interfere with lipid bilayer and alter the aquaporin transport in adipocytes, specifically through AQP7. This aquaporin is also expressed in adipose cells and has the capacity to act like a glycerol pressure valve, letting some of the glycerol enter in bloodstream (Frühbeck, 2005). If the AQP7 is missing or dysfunctional, glycerol accumulates in the fat cells, driving to additional synthesis of fat.

- Additionally to *c*9,*t*11 and *t*10,*c*12 CLA isomers, so well studied, through *in vitro* and *vivo* studies, other CLA isomers are consumed which biological effects are still unknown. For example, it would be interesting to investigate the potential health effects of the *t*7,*c*9 and *t*11,*c*13 isomers that may reach ~20% of total CLA in food products from ruminant origin (Martins *et al.*, 2007). The chemical structure-activity relationship suggests that a system of conjugated double bonds is required for the biological function of these isomers. This hypothesis was already validated for long chain fatty acids with 19, 20 and 22 carbons of synthetic origin (Park & Park, 2009). As so, one can hypothesised about *t*7,*c*9 and *t*11,*c*13 beneficial properties, as well, having these isomers the advantage of being natural compounds. This study implies additional work to isolate, purify and produce these CLA isomers. Informatic modelation based on structure-activity, using the *t*10,*c*12 isomer as model, could also be an interesting tool in order to select the CLA isomers with the most putative anti-adipogenic effects.

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